Effects of hormones and salinity on branchial Na^+-K^+-ATPase expression in the sea bream, *Sparus sarba*

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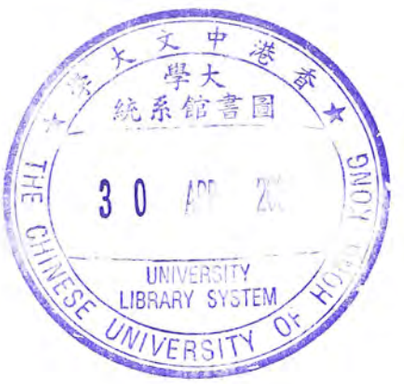
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摘要

這論文討論了鯛魚(*Sparus sarba*)鰓部鉀鈉腺苷三磷酸酶的表達。實驗分爲兩部份，第一部份的研究是體外實驗，正常鯛魚的鰓分別接受不同濃度的激素處理。所用的激素包括重組鯛生長激素(rbGH)、重組鯛類胰島素生長因子-I(rbIGF-I)、綿羊催乳激素(oPRL)及皮質醇。第二部份的研究是體內實驗，收集鯛魚適應於不同鹽度的鰓部作研究。激素與鹽度對鰓部鉀鈉腺苷三磷酸酶的轉錄、轉譯及後轉譯過程的影響，可於處理後的鯛魚鰓部鉀鈉腺苷三磷酸酶的α-, β-核糖核酸, α-蛋白及酵素活性的改變中顯示。

在第一部份實驗裏，實驗目的是研究激素在體外對鯛魚鰓部鉀鈉腺苷三磷酸酶表達的作用。鯛魚的鰓持續兩小時接受不同濃度的體外激素處理，結果顯示重組鯛生長激素於較低濃度(1ng/ml)時能明顯地上調鰓部鉀鈉腺苷三磷酸酶α-及β-核糖核酸的水平；較高濃度的處理，有趨勢以劑量依賴方式降低其水平，但改變並不顯著。各劑量的重組鯛類胰島素生長因子-I 都能顯著地上調鉀鈉腺三磷酸酶α-核糖核酸的水平，以低劑量最能誘導其水平。但重組鯛類胰島素生長因子-I 卻以劑量依賴方式明顯地降低β-核糖核酸的水平。另外，結果顯示綿羊催乳激素或皮質醇不論對鯛魚鰓部鉀鈉腺苷三磷酸酶α-或β-核糖核酸的表達都沒有明顯的影響。

在激素對鯛魚鰓部鉀鈉腺苷三磷酸酶α-蛋白體外實驗內，重組鯛生長激素及重組鯛類胰島素生長因子-I 於較低濃度(1,10ng/ml)時都能明顯地上調鯛魚鰓鉀

鈉腺苷三磷酸酶α-蛋白的表達，兩者都以 1ng/ml 的劑量的影響最爲顯著，分別較對照高 2.3 及 6.6 倍。綿羊催乳激素或皮質醇則對鯛魚鰓部鉀鈉腺苷三磷酸酶α-蛋白的表達沒有明顯的影響。

激素對鯛魚鰓部鉀鈉腺苷三磷酸酶的酵素活性影響實驗內發現，隨著重組鯛生長激素或重組鯛類胰島素生長因子-I 的劑量增多，酵素活性呈拱形影響。重組鯛生長激素於 10ng/ml 令該酵素活性顯著上升，1 及 10mg/ml 重組鯛類胰島素生長因子-I 處理後，都能明顯地上調該酵素活性。綿羊催乳激素或皮質醇對鯛魚鰓部鉀鈉腺苷三磷酸酵素活性的表達都沒有明顯的影響。

第二部份的研究針對廣鹽性的鯛魚鰓部鉀鈉腺苷三磷酸酶的表達在不同鹽度下的變化。首先，鯛魚適應於四個不同的鹽度，四星期後，量度鰓部鉀鈉腺苷三磷酸酶的α-, β-核糖核酸, α-蛋白及酶活性的水平改變。四個鹽度包括高滲鹽度(50 ppt)、正常海水鹽度(33 ppt)、等滲鹽度(12 ppt)和低滲鹽度(6 ppt)。結果顯示鉀鈉腺苷三磷酸酶β-核糖核酸在高滲鹽度(50 ppt)及正常海水鹽度(33 ppt)顯著地高於等滲鹽度(12 ppt)或低滲鹽度(6 ppt)。適應於正常海水鹽度(33 ppt)或等滲鹽度(12 ppt)的鰓部鉀鈉腺苷三磷酸酶α-蛋白明顯高於低滲鹽度(6 ppt) 環境下的表達，而以高滲鹽度(50 ppt) 環境下適應的鯛魚鰓部鉀鈉腺苷三磷酸酶α-蛋白最爲高。於測試該酵素活性的實驗內，適應於高滲鹽度(50 ppt) 環境下的鯛魚鰓部鉀鈉腺苷三磷酸酵素活性最高。

結果說明了生長激素和類胰島素生長因子-I 能有效地直接促進或穩定鯛魚鰓部鉀鈉腺三磷酸酶的轉錄、轉譯及後轉譯的表達，亦推斷出綿羊催乳激素及皮質醇對鯛魚鰓部鉀鈉腺苷三磷酸酶的表達沒有直接明確的影響。此外，實驗結果亦說明了高鹽度環境上調鰓部鉀鈉腺苷三磷酸酶的表達。鉀鈉腺三磷酸酶的β-部分是一合成有功能性的鉀鈉腺三磷酸酶的必然要素。

Abstract

In this thesis, the Na^+-K^+-ATPase expression in the branchial tissues of *Sparus sarba* was studied. The experiments consisted of two parts. In the first part, the Na^+-K^+-ATPase expression in branchial tissues in response to *in vitro* hormone treatments was investigated. The hormones used included recombinant bream growth hormone (rbGH), recombinant bream insulin-like growth factor-I (rbIGF-I), ovine prolactin (oPRL) and cortisol. In the second part, fish were subjected to *in vivo* osmotic stress and branchial Na^+-K^+-ATPase expression was then investigated. Through these approaches, the effects of hormones and salinity on branchial Na^+-K^+-ATPase expression can be demonstrated at the transcriptional, translational and posttranslational levels through changes in the levels of Na^+-K^+-ATPase mRNA, protein subunits and enzyme activity respectively.

In the first part of the thesis, the aim of the study was to establish whether key hormones directly regulate *Sparus sarba* branchial Na^+-K^+-ATPase expression. After *in vitro* incubation of branchial arches for 2 hours at different concentrations of various hormones, the results demonstrated that 1ng/ml rbGH upregulated *Sparus sarba* branchial Na^+-K^+-ATPase α and β mRNA subunit levels. Higher concentrations of rbGH treatment resulted in a trend of decreasing mRNA subunit levels but the changes were not significant. All concentrations of rbIGF-I stimulated α-mRNA subunit expression and a dose of 1ng/ml rbIGF-I was the most effective. However, rbIGF-I depressed branchial Na^+-K^+-ATPase β mRNA subunit expression. oPRL and cortisol were found to have no significant effect on branchial Na^+-K^+-ATPase mRNA as well as α or β mRNA subunit expression.

In the investigation of hormonal effect on *Sparus sarba* branchial Na^+-K^+-ATPase α-subunit protein, both rbGH and rbIGF-I upregulated Na^+-K^+-ATPase α-subunit protein expression at doses of 1 and 10ng/ml. The effect was most marked at 1ng/ml in which a 2.3 fold and 6.6 fold stimulation was observed for rbGH and rbIGF-I respectively. However, oPRL and cortisol showed no significant effect on *Sparus sarba* branchial Na^+-K^+-ATPase α-subunit protein.

In the experiment on the investigation of hormonal effect on branchial Na^+-K^+-ATPase activity, the results showed that as the doses of rbGH or rbIGF-I were increased, Na^+-K^+-ATPase activity expression changed in a bell-shaped manner. Doses of 10ng/ml rbGH and 1,10ng/ml rbIGF-I increased Na^+-K^+-ATPase activity significantly. However, oPRL and cortisol caused no significant effect on *Sparus sarba* branchial Na^+-K^+-ATPase activity.

In the second part, the effect of salinity on Na^+-K^+-ATPase expression in branchial tissues of *Sparus sarba* was examined *in vivo*. Firstly, *Sparus sarba* was subjected to 4 different salinity levels such as hypersaline condition (50ppt), normal seawater (33ppt), iso-osmotic condition (12ppt) and hypoosmotic condition (6ppt). *Sparus sarba* branchial Na^+-K^+-ATPase mRNA, protein subunits expression and enzyme activity were then investigated. Branchial Na^+-K^+-ATPase β-subunit mRNA were significantly higher in fish acclimated to 50ppt and 33pt than those acclimated to 12ppt and 6ppt. 33ppt or 12ppt acclimated *Sparus sarba* had higher branchial Na^+-K^+-ATPase α-subunit protein than that acclimated to 6ppt. 50ppt acclimated *Sparus sarba* showed the highest Na^+-K^+-ATPase α-subunit protein level and enzyme activity.

The results indicated that GH and IGF-I can directly upregulate or stabilize *Sparus sarba* branchial Na^+-K^+-ATPase transcriptional, translational and posttranslational processes and showed that oPRL and cortisol had no direct effect on *Sparus sarba* branchial Na^+-K^+-ATPase expression. Furthermore, it also indicated that hyperosmotic condition elevated *Sparus sarba* branchial Na^+-K^+-ATPase expression, and Na^+-K^+-ATPase β-subunit is essential for the functioning of Na^+-K^+-ATPase.

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Chapter 1

General introduction

Through effective mechanisms of osmoregulation, teleosts are able to maintain osmotic constancy of internal milieu and survive in hypertonic seawater and hypotonic fresh water. The principles of osmoregulation and adaptation of teleosts to environments of different salinities are generally accepted to involve concerted responses controlling the rate of drinking and the subsequent regulation including the modulation of ion transport in the secretory or absorptive epithelia of the osmoregulatory organs such as gill, intestine, liver, kidney and skin surface. These cause changes in serum chemistry, muscle moisture, branchial, renal, hepatic and intestinal enzyme activities etc. Through these processes, teleost fishes maintain their plasma osmotic concentration at a constant level irrespective of several orders of magnitude changes of salinity of their external environments.

In 1984, Evans has estimated that 95% of teleost species are stenohaline, living in either fresh water or seawater. The remaining 5% are euryhaline, having the capacity to withstand large changes in environmental salinity. The transition between a freshwater hyperosmoregulator and a seawater hypoosmoregulator necessitates an alteration in osmoregulatory mechanisms. Teleosts show a wide spectrum of morphological and physiological adaptation to waters of different osmolarity and ionic composition. Teleost hypoosmoregulate in seawater and balance the influx of ions and the efflux of water by drinking seawater (Usher *et al.*, 1988), absorbing water and salts across the gut (Simmoneaux *et al.*, 1987), and secreting excess monovalent ions across the gills (Borgatti *et al.*, 1992) and divalent ions through the

kidney (Brown *et al.*, 1980). In freshwater, the fish must counteract the continually passive gain of water and loss of ions across permeable body surfaces. They balance the influx of water by producing large quantities of dilute urine through the renal system. Salt losses from the body are reduced by lowering the body surface permeability and actively take up ions across the gill, from the food in the intestine and an efficient ion reabsorption from the kidney (Foskett *et al.*, 1983; Perrott *et al.*, 1992). Recent reports of marine fish hyperosmoregulation has been demonstrated by various research groups (Mancera *et al.*, 1993b; Woo & Kelly, 1995; Morgan *et al.*, 1996; Kelly *et al.*, 1999b, c; Kelly &Woo, 1999a), and the ability of marine fish to actively hyperosmoregulate in dilute media is now thought to be widespread (Wu and Woo, 1983). Woo and Chung (1995) coined the term "physiological euryhalinity" to indicate a widespread euryhalinity among otherwise "ecologically stenohaline" marine fish species.

Gill is one of the major sites of ion movements to balance diffusional gains or losses. Reports implicate the chloride cell as the main site of ion transport where ion-transporting enzymes are located and contact with both blood and water (Perry, 1997; Fernandes *et al.*, 1998). Ion transport mechanisms are complex, involving several transporters. Na^+-K^+-ATPase, with high levels (more than 10^8 in each chloride cell) localized on the basolateral membrane of the branchial epithelial cells, is a membrane-spanning protein and the main driving force of many transport systems across animal cell membranes by generating an electrochemical gradient (Karnaky *et al.*, 1976; Hootman and Philpott, 1979; Ernst *et al.*, 1980; Karnaky, 1986; McCormick, 1990). Therefore the regulation of Na^+-K^+-ATPase expression in the

organs is of major importance to fish during both freshwater and seawater acclimation (Payan *et al.*, 1984).

Na^+-K^+-ATPase is mainly regulated by environmental factors such as salinity and endocrine factors. Hormones have definite regulatory role on enzymes of osmoregulation in the teleost. GH, IGF-I, PRL are peptide hormones and cortisol is steroid hormone which responsible for adaptation of teleosts to different salinities (Hirano, 1986; Sakamoto *et al.*, 1993; McCormick, 1995). Specifically, studies indicate that the growth hormone/insulin-like growth factor I axis and cortisol are seawater adapting hormonal systems which increase the hypo-osmoregulatory ability of the seawater adapted teleosts (Yada and Hirano, 1992; Sakamoto and Hirano, 1993; McCormick, 1995; Wendelaar Bonga, 1997; Seidelin *et al.*, 1999; Leena and Oommen, 2000). On the other hand, prolactin is the freshwater adapting hormone, and increases the hyper-osmoregulatory ability in freshwater adapted fish (Brown and Brown, 1987; Bern and Madsen, 1992; Mancera *et al.*, 1993a; Kelly *et al.*, 1999c; Tang *et al.*, 2001). Recent studies have shown that some of the hormones and different salinity levels have some effect on Na^+-K^+-ATPase expression, particularly at the enzyme activity level, however, the regulation of such factors on the mRNA and protein subunits of Na^+-K^+-ATPase is still not known. In this study, the hormonal and salinity effects on Na^+-K^+-ATPase expression in the gills of silver sea bream or gold-line sea bream (*Sparus sarba*) were examined. This study aims to clarify the confusing issues concerning interspecific differences in the relative contributions of hormones to osmoregulation.

In view of the prominent physiological role played by Na^+-K^+-ATPase, understanding of how it is regulated in response to changing physiological demand is a major concern in cell biology. As the regulation of Na^+-K^+-ATPase is multihormonally controlled, the actual effect of each hormone on Na^+-K^+-ATPase is still not clearly known. Therefore, an *in vitro* approach is adopted in the present study. The first objective of this study was to investigate whether some key hormones directly regulate gill Na^+-K^+-ATPase expression *in vitro*. In this experiment, gill of *Sparus sarba* was incubated in medium with four hormones individually: recombinant bream growth hormone (rbGH), recombinant bream insulin-like growth factor-I (rbIGF-I), ovine prolactin (oPRL) and cortisol (F). Other than the hormones, the most widely recognized and investigated environmental determinant of gill Na^+-K^+-ATPase and chloride cell development is salinity. Isoosmotic condition is believed to be the minimum stress environment which is optimal for fish survival and culture practice as growth rates and protein efficiency ratios of *Sparus sarba* were higher at 15ppt than those at 7 or 35 ppt (Woo and Kelly, 1995). Another aim of this study is to investigate the effect of salinity stress on gill Na^+-K^+-ATPase expression in euryhaline teleost *Sparus sarba in vivo* to test the hypothesis that iso-osmotic environment as an osmoregulatory energetics indicator. Silver sea bream was acclimated to various salinities including 50 ppt (hyper-osmotic salinity), 33 ppt (normal seawater), 12 ppt (iso-osmotic salinity of silver sea bream), 6 ppt (hypo-osmotic salinity) for more than 1 month and the expression profile of branchial Na^+-K^+-ATPase assessed.

Chapter 2

Literature review

2.1. Gill

Gills are important sites for gas exchange, acid-base regulation and ion exchange in fish and are the most important extra-renal organs responsible for osmoregulation in both seawater and freshwater adapted fish. Fish gills consist of two distinct epithelial surfaces, the lamellar and filament epithelia. There are a large number of gill filaments arranged along the gill arches on each side of the branchial basket. The surfaces of the filaments are greatly enlarged by secondary lamellae. The morphological attributes of the fish gill which define its usefulness in gas exchange are its highly vascularised, thin epithelium with a large surface area. Other than that, gills are surrounded by an aquatic medium and their selectively permeability allow many soluble substances to pass across the gill epithelia. It is the site where the majority of net water and salt movements take place if the organism is in an aquatic environment whose osmotic and ionic concentrations differ from those of the body fluids (Perry, 1997; Karnaky, 1998; Evans *et al.*, 1999). The gill epithelium contains four distinct cell types including chloride cells or also called mitochondria-rich cells, pavement cells or otherwise termed respiratory cells for respiration, mucous cells, and neuroepithelial cells (Perry, 1997; Van Der Heijden *et al.*, 1997). Gill epithelium generally serves diverse functions such as respiration, acid-base regulation and ion regulation. Any transporting cell has basolateral (serosal, blood side) and apical (mucosal, medium side) membranes that divide the transport pathway into three (medium, cytoplasm and blood) compartments separated by two barriers.

2.2. Chloride cells (Mitochondria-rich cells)

In most teleost species that have been examined under normal conditions, the chloride cells occupy only a small fraction, generally less than 15% of the total surface area of all epithelia cells exposed to the environment. They are well known to be responsible for osmoregulation in fish which play a vital role in ionic regulation in both hypo-osmotic and hyper-osmotic environments. It is generally accepted that the chloride cells are the site of ion absorption such as Cl^- and Ca^+ and acid-base regulation in freshwater-adapted fish (Avella *et al.*, 1987; Laurent, 1989; Laurent & Habibi, 1989; Laurent & Perry, 1990; Perry & Laurent, 1993). In the opercular membrane of seawater acclimated teleosts, chloride cells comprise the cellular sites for the excretion of excess monovalent ions, sodium and chloride (Foskett & Scheffey 1982; Hwang 1987; Perry et al. 1992). The osmoregulatory and metabolic strategies at the gill are specially accentuated with alterations in branchial chloride cell numbers and surface ultrastructural morphometrics. Branchial chloride cells increase in apical and fractional exposure area after low salinity exposure (Kelly *et al.*, 1999a).

In seawater, the chloride cells are relatively abundant on the filamental epithelium but appear on the lamellar epithelium in freshwater adapted species suggesting chloride cells located on the filaments may be involved in ion secretion whereas those on the lamella may be involved in ion absorption (Laurent & Dunel 1980; Perry and Wood, 1985; Avella *et al.*, 1987; Uchida *et al.*, 1996; Perry, 1997; Hirai *et al.*, 1999). Filamental chloride cells were larger and more closely spaced than those in the lamellae (Choe *et al.*, 1999).

Chloride cells were also termed "mitochondria-rich cells", the name indicating these highly specialized cells are characterized by numerous mitochondria with relatively high metabolic activity compared to pavement cells (Perry and Walsh, 1989). They possess a large and columnar appearance, an apical crypt, mucosal-serosal exposure and contain sub-apical vesicles with an extensive tubular system and anatomizing network of tubules which is continuous with the basolateral membrane, resulting in a large surface area for the placement of transport proteins (Evans *et al.*, 1982; Zadunaisky, 1984). There is substantial evidence indicating the biochemical mechanisms for maintenance of constant levels of ions in body fluids of fish depend on the activity of the major transporter, Na^+-K^+-ATPase which is basolaterally located, and the enzyme activities of gills in euryhaline teleost are affected by environmental ion concentrations (Hwang *et al.*, 1989; McCormick 1995; Kelly *et al.*, 1999a, b). This enzyme is mainly located in the tubular system of the chloride cell and may be up to 10^8 Na^+-K^+-ATPase molecules present in a single chloride cell which can be used as a cellular marker for differentiating branchial epithelium chloride cells from pavement cells (Karnaky *et al.* 1976; Uchida *et al.*, 1996; Karnaky, 1986; Perry, 1997).

Pisam and coworkers (1987) identified two subpopulations of chloride cell (α and β cells) on the primary lamellae of freshwater species and euryhaline species in freshwater on the basis of their location, shape and ultrastructure. The α chloride cells are characterized as elongated cells, with extensively folding and projections, which located at the base of the secondary lamellae in close contact with the basement membrane of the pillar capillary, are strongly Na^+-K^+-ATPase immunoreactive and are thought to be the precursors or homologue of the characteristic chloride cells seen

in seawater adapted fish. On the other hand, the β chloride cells are characterized as smooth, ovoid cells observed in the interlamellar regions facing the central venous sinus, are weakly immunoreactive with Na^+-K^+-ATPase antibodies and only exist in freshwater teleosts. The study also reported that the α chloride cells hypertrophied whereas the β chloride cells degenerated when the fish were transferred to seawater, suggesting that the α chloride cells and β chloride cells play significant roles in seawater and freshwater adaptation respectively. An increase in chloride cell size and density has been reported in the gills of adult teleosts transferred from freshwater to seawater (Pisam and Rambourg, 1991). Morphological changes in the gill chloride cells were induced by environmental salinity, indicating that chloride cell is multifunctional (Dange 1985; Hwang, 1987; Fernandes *et al.*, 1998; Kelly *et al.*, 1999a; Kelly and Woo, 1999a).

Chloride cells were classified into three developmental stages: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex of chloride and accessory cells with an apical pit (Hiroi *et al.*, 1999). The single chloride cells without pits are probably immature cells, since they are not exposed to the external environment and are smaller in size. However, their strong cytoplasmic Na^+-K^+-ATPase immunoreactivity indicates that the tubular system has already developed because Na^+-K^+-ATPase is located in this system. These immature chloride cells may originate from undifferentiated cells with a nucleus similar to that of chloride cells, and then enlarge their cytoplasm and come into contact with the ambient water by forming apical pits. Accessory cells are thought to be young stages of chloride cells (Hootman and Philpott, 1980; Wendelaar Bonga and van der Meij, 1989). If accessory cells were young chloride cells, they

would develop further and replace the main chloride cells when the main chloride cells degenerate. The single chloride cells are ready to function as ion-transporting cells after form apical pits. The multicellular complex generally consists of one main chloride cell and one (rarely two) accessory cell. Two or more chloride cells of similar size share one pit, and this type of complex is also associated with one or two accessory cells. All three subtypes of chloride cell are found in both freshwater and seawater fish, the number of multicellular complexes increases in number after seawater transfer, whereas they decrease in number in freshwater (Hiroi *et al.*, 1999).

2.2.1. Ion extrusion by fish in seawater

In seawater adapted fish, the osmoregulatory function of the gill is to secrete excess monovalent ions and the chloride cells of marine fish are essential for eliminating excess Na^+ and Cl^- due to the fact that their basolateral membranes contain the enzyme Na^+-K^+-ATPase (McCormick, 1995). Na^+-K^+-ATPase generated an electrochemical gradient for the transport of Na^+ from the plasma to the cytoplasm of the chloride cell, which drove the secretion of Na^+, Cl^-, K^+, H^+ and NH_4^+ and the absorption of Ca^{2+} (Zadunaisky, 1984; Foskett and Machen, 1985; Marshall and Bryson, 1998). A recent model for the maintenance of ion balance in seawater fish is best decribed in a review by Marshall (1995) (Fig. 2.1). Na^+-K^+-ATPase creates low intracellular Na^+ and a highly negative charge within cell by driving 3 Na^+ out and 2 K^+ into the cell across the chloride cell basolateral membrane. The Na^+ gradient is then used to transport Cl^- into the cell through a $Na^+/K^+/Cl^-$ cotransporter. Cl^- leaves the cell "downhill" on an electrical gradient through an apical Cl^- channel. Na^+ is transported down its electrical gradient (seawater being more negative in

electrochemical potential than plasma) through a leaky paracellular pathway that lies between the accessory cell and mitochondria-rich cell (Wood and Marshall, 1994). The processes involve ouabain sensitive basolateral Na^+-K^+-ATPase (Sweadner, 1989), furosemide sensitivity basolateral $Na^+/K^+/Cl^-$ cotransporter (Payne and Forbush, 1995; Kaplan *et al.*, 1996), basolateral and apical K^+ channel and apical Cl^- channel (Marshall and Bryson, 1998).

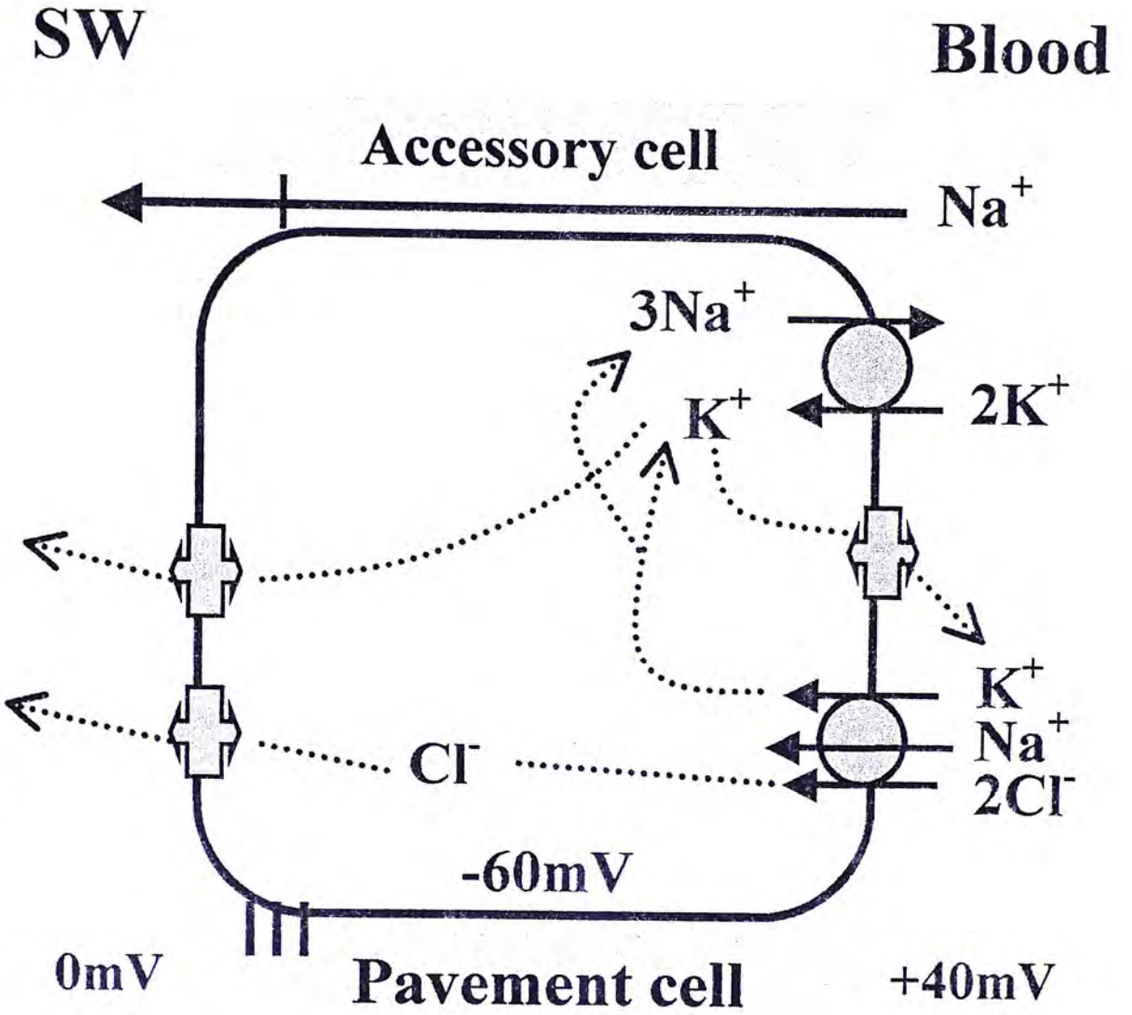


Fig. 2.1 Current model for NaCl excursion by the seawater adapted fish chloride cell. Note the deep-tight junction between the chloride cell and pavement cell, and shallow-tight junction between the chloride cell and accessory cell. (Redrawn from Marshall, 1995)

2.2.2. Ion uptake by fish in hypo-osmotic conditions

Fish continually lose ions across permeable body surface and extensive gill epithelial surface. The large electrochemical gradients for both Na^+ and Cl^- between fish plasma and the medium in freshwater dictate that mechanisms must exist for extraction of both of these ions from the very dilute environment. Several reports showed that extrusion of NH_4^+, H^+ or HCO_3^- was probably chemically coupled to Na^+ and Cl^- uptake in order to maintain reasonable electrical gradients across the gill (Evans, 1975; McDonald *et al.*, 1989).

There are several models for ion movement across the gill (Evans *et al.*, 1982; Lin and Randall, 1991, 1995; Potts, 1994; Perry, 1997). Intracellular generation of the H^+ and HCO_3^- in order to ion transport is probably from hydration of CO_2. The hydration process is done by carbonic anhydrase within the chloride cell (Lacy, 1983). Na^+ enter the gill epithelia by exchange with H^+ by an electroneutral, amiloride-sensitive Na^+/H^+ exchange protein, or alternatively through an apical amiloride-sensitive epithelial Na^+ channel coupled to and energized by an apical H^+-ATPase. The active transport of Na^+ into the cell may also be driven by the transport of NH_4^+. Intracellular Na^+ then was pumped into the blood at the basolateral surface through Na^+-K^+-ATPase. The transport of the HCO_3^- can also be extruded into the external environment in exchange for Cl^- by an apical electroneutral Cl^-/HCO_3^- anion-exchange protein. Cl^- then leaves at the basolateral membrane moving 'downhill' on an electrical gradient through its channel (Wilson *et al.*, 2000) (Fig. 2.2).

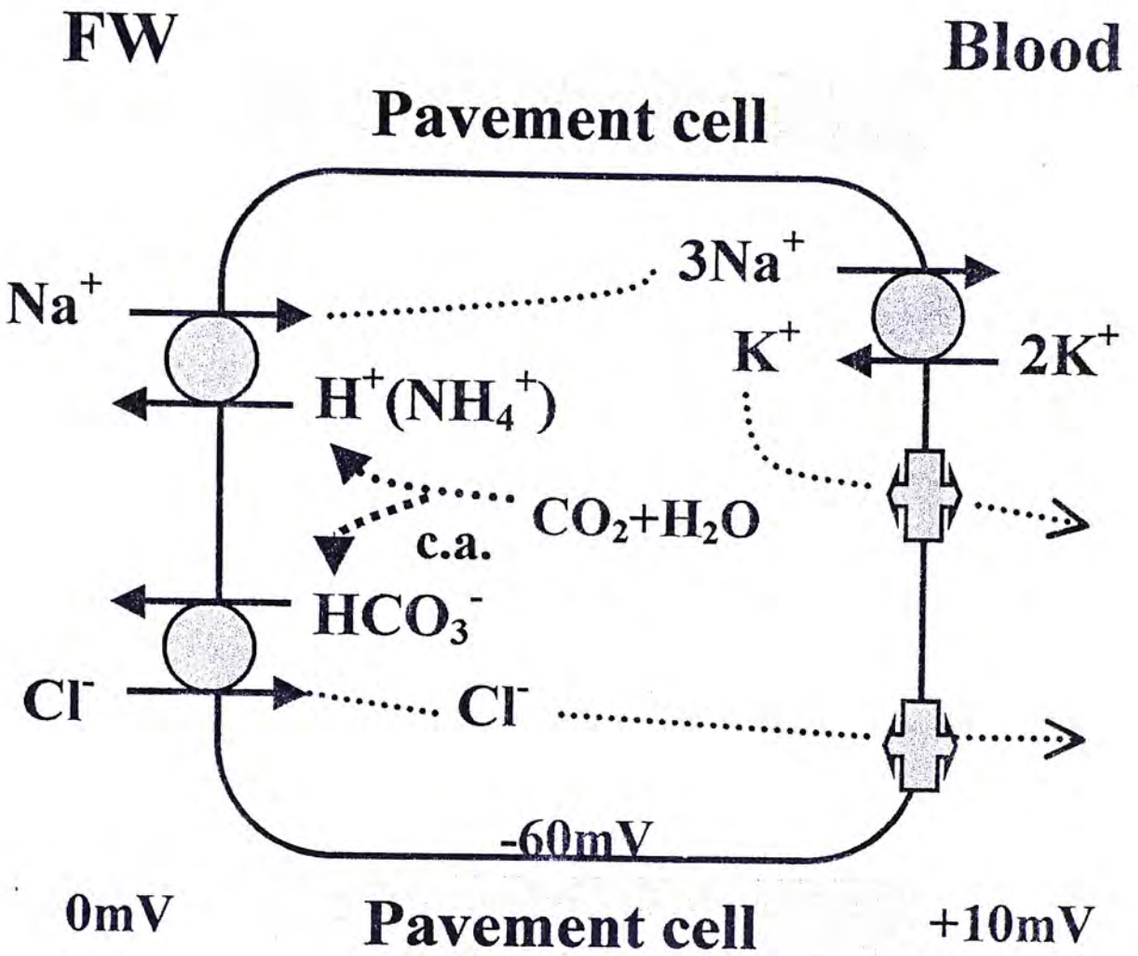


Fig. 2.2 Current model for NaCl extrusion by the freshwater adapted fish chloride cell. c.a. indicates carbonate anhydrase (Redrawn from Marshall, 1995)

2.3. *Sparus sarba* (Silver seabream)

Members of the Sparidae commonly referred to as seabream are generally considered "true" marine fish as they are never found in hypo-osmotic condition and do not appear to require a fresh water environment for any part of their life cycle (Woo and Wu, 1982; Woo and Kelly, 1995). Silver seabream (*Sparus sarba*) is a member of the Sparidae. These fish, however, do frequent estuarine environments and, are likely to experience a degree of salinity variation, which necessitates some efficient osmoregulatory strategies on the regulation of salts during natural exposure to salinity fluctuation. The sparids (Kelly *et al.*, 1999b; Kelly, 1997; Wong, 2001) have been shown to tolerate a wide salinity range from 0-50 ppt. Although they do not migrate between freshwater and seawater like the salmonids, they can be found in tropical and sub-tropical estuaries, where they are considered to be marine migrants as they experience salinity fluctuation during estuarine entry (Sheaves *et al.*, 1999). Moreover, the culture of certain species of marine fish in salinity regimes lower than seawater result in enhanced growth (Woo and Kelly, 1995).

2.4. Sodium-potassium adenosinetriphosphatase (Na^+-K^+-ATPase)

Na^+-K^+-ATPase, or sodium pump, is the essential enzyme in all eukaryotic cells of higher animals (Rossier *et al.*, 1989; Skou and Esmann, 1992). It is responsible for the creation and preservation of the ion-selective concentration and electrochemical gradients of high K^+ and low Na^+ intracellular concentration across the plasma membrane by utilizing the energy of the hydrolysis of ATP to ADP (Sweadner, 1989). The enzyme modulates Na^+ balance by coupling the active efflux of three Na^+ and influx of two K^+ to the cell. It provides the necessary energy for the proper functioning of many Na^+-dependent transport systems to move, e.g. phosphate, amino acids, or glucose into the cell (Charnock and Simonson, 1977; Ferraris and Ahearn, 1983; Lorenzo *et al.*, 1987,1989) or to remove protons or Ca^{2+} from the cells (Wilson *et al.*, 2000) and also some specialized functions such as the regulation of intracellular pH, regulation of cell volume, the propagation of nerve impulses, muscle contractions uptake of nutrients, etc (Blanco and Mercer, 1998). It plays a central role in the process of ion transport in gills of freshwater-and seawater-adapted fishes and participates both directly through movement of sodium and potassium across the plasma membrane and indirectly through generation of ionic and electrical gradients (Silva *et al.*, 1977; Avella and Bornancin, 1989).

The enzyme belongs to a multigene family and comprises two noncovalently linked polypeptides, α- and β- subunits. Each subunit spans the lipid bilayer of the cell membrane (Geering, 1990). Recently, with the development of new molecular cloning, sequencing and immunological techniques, Na^+-K^+-ATPase is found to be composed of several clearly different subunit types which with different biological

functions. The gene expression of each subunit is differentially regulated in various tissues and developmental stages (Okamoto and Kawakami, 1992). A proteolipid γ-subunit has also been found to copurify with the Na^+-K^+-ATPase and, although not essential for enzyme activity, may modify enzyme function.

Several processes are involved in biosynthesis and membrane insertion of α- and β-subunits of Na^+-K^+-ATPase. Synthesis is initiated on free ribosomes in the cytoplasm. Both α- and β-subunits are coordinately synthesized and inserted into membrane of the endoplasmic reticulum during their translation. The abundance of Na^+-K^+-ATPase in gill epithelia of most euryhaline fish is adaptively changed when the external salinity is changed that the process is controlled largely by hormonal modulation of gene and protein expression. Some studies reported that rapid modulation of gill Na^+-K^+-ATPase activity are the result from salinity change (Mancera and McCormick, 2000), the activation of protein kinase C (PKC) (Crombie *et al.*, 1996) and the activation of cyclic-AMP-mediated protein kinase A (PKA) (Marver *et al.*, 1986). Protein kinases and protein phosphatases play a crucial role in the modulatory event. Several possible mechanisms for the regulation of the Na^+-K^+-ATPase by PKA have been proposed including trafficking between endosomes and the basolateral membrane (Carranza *et al.*, 1998), activation of arachidonic acid metabolism (Satoh *et al.*, 1992) and direct phosphorylation of the enzyme, affecting its activity (Cheng *et al.*, 1997). The use of mannitol for increasing osmolality of *in vitro* culture medium without increasing external Na^+ levels also induced a rapid activation of Na^+-K^+-ATPase activity and increased osmolality itself can induce rapid activation of gill Na^+-K^+-ATPase (Mancera and McCormick, 2000).

2.4.1. Na^+-K^+-ATPase α-subunit

Throughout the animal kingdom, in diverse vertebrate and invertebrate species, the amino acid sequence of the α-subunit is extremely well conserved (Shull *et al.*, 1985; Kawakami *et al.*, 1985; Takeyasu *et al.*, 1989, 1990; Blanco and Mercer, 1998). Three isoforms of α-subunit (α1, α2 and α3) were found in the rat and chicken (Takeyasu *et al.*, 1990; Blanco and Mercer, 1998). These α-subunit isoforms show tissue-specific and developmentally dependent patterns of expression in birds and mammals and this was suggested to extend to all vertebrate classes, including teleosts (Takeyasu *et al.*, 1990; Pressley, 1992). Cloning and sequence studies of the α-subunit demonstrated about 80% similarity between the α-subunit of animals such as *Drosophila*, fish, amphibia, birds and mammals. The fragments obtained from the PCR product showed a sequence corresponding to the α-subunit of the Na^+-K^+-ATPase as a heterogenous population of 3 sequences with 86-93% identity when compared among each other (D' Cotta *et al.*, 1996). This suggests that different allelic forms of the α-subunit are expressed in gill tissue.

At the protein level, the α-subunit has several transmembrane segments and a large cytoplasmic loop containing the phosphorylation site and the ATP binding site. Three isoforms of Na^+-K^+-ATPase α-subunit have been identified which differ, among other characteristics, in their tissue distribution, sensitivity to cardiac glycosides, affinity for Na^+ and K^+, and conformational equilibrium between the E_1 and E_2 state and in hormonal regulation. The catalytic α-subunit, a molecule consists of 1022 amino acids and has a calculated molecular weight of about 100 to 112 kDa and six to eight membrane-spanning domains (Kawakami *et al.*, 1985). Among the 3

isoforms, α1 is the form that is predominantly expressed in the transporting epithelia in higher vertebrates (Sweadner 1989; Takeyasu *et al.*, 1990; Lingrel 1992; Pressley 1992). Na^+-K^+-ATPase α-subunit contains most of the functional domains and carries all functional properties of the enzymes, such as the ability to hydrolyze ATP, to bind Na^+ and K^+ or else glycosides, specific inhibitors of Na^+-K^+-ATPase. The α1-subunit functions primarily in a housekeeping capacity to maintain osmotic balance and cell-volume regulation while the other α-subunits fulfill more specific requirements for cation transport necessary for differentiated cell-specific functions (Blanco and Mercer, 1998). In the human genome, at least five different sequences related to the Na^+-K^+-ATPase α-subunit have been detected and partially characterized.

2.4.2. Na^+-K^+-ATPase β-subunit

The β-subunit, on the other hand, is a glycoprotein, and has a short cytoplasmic tail, with one transmembrane segment and a large extracytoplasmic domain containing three to four glycosylation sites. The β-subunit has a molecular weight of approximately 35 to 55 kDa and is a glycoprotein of 300 amino acids with a single membrane-spanning domain. Three β subunits (β1, β2 and β3) isoforms have been described so far (Geering, 1990; Lingrel, 1992). The amino acid sequence of the β-subunit has about 61% similarity between the β-subunit of fish and mammals (Noguchi *et al.*, 1986). Comparing the similarity of the β-subunit amino acid sequence among vertebrates, it is high (over 90%) within mammalian species but decreases in amphibians (65% similarity with mammalian β-subunit) or fish (61% similarity with mammalian β-subunit). The amino acid sequence is less highly

conserved among vertebrates (60%) (Rossier *et al.*, 1989; Cutler *et al.*, 2000). Level of the β_1 isoform-mRNA increased in gills, kidney and intestine during seawater acclimation, suggesting that β_1 isoform of Na^+-K^+-ATPase may play an important functional role in the major osmoregulatory tissue of hyperosmoregulatory fish.

The electrophoretic mobility of the β-subunit from different tissues differ on SDS-polyacrylamide gels but the same β-subunit form is expressed in restriction mapping and DNA sequencing in different tissues suggesting that the apparent difference in the molecular mass of the glycosylated β-subunit in different tissues was mainly due to tissue-specfic differences in oligosaccharide (Geering, 1990).

Na^+-K^+-ATPase β-subunit has not yet been assigned a definite role, though its presence has been identified in crystalline Na^+-K^+-ATPase but the interaction between the α- and the β-subunit might be important in the hydrolytic function of the Na^+-K^+-ATPase. Some studies suggested the β-subunit is also necessary for Na^+-K^+-ATPase functioning. Schmalzing and Gloor (1994) suggested that β-subunit stabilizes the correct folding of the α-subunit, delivers the alpha subunit to the plasma membrane, confers K^+ affinities, modulates enzyme affinities for ion substrates and is involved in cell-cell interactions. Reduction of a disulphide bond between Cys 158 and Cys 175 of the β-subunit resulted in loss of enzyme activity of the purified enzyme (Kawamura and Nagano, 1984). β_3 isoform transcript was only detected in *Anguilla anguilla* brain but not in key osmoregulatory organs such as intestine, kidney and gill indicating the major roles of β_3 isoform is other than osmoregulation such as neuronal interactions (Cutler, 2000).

2.4.3. Regulation of Na^+- K^+-ATPase

There are many factors affecting and regulating Na^+- K^+-ATPase expression. The regulation of Na^+-K^+-ATPase is mainly affected by endocrine factors and environmental factors such as salinity. Gill Na^+-K^+-ATPase expression was also affected during spawning period, with reduced enzyme activity in normal gonadal development of female brook charr as compared to sterile fish (Le Francois and Blier, 2000). Other factors such as synethetic steroid dexamethasone (DEX) provoked a significant increase in chloride cell Na^+-K^+-ATPase activity in both seawater and freshwater adapted fish (Marsigliante *et al.*, 2000). Na^+-K^+-ATPase is also very sensitive to heavy metal inhibition such as water-borne copper (Cu^{2+}) (Li *et al.*, 1998) and silver (Ag^+) (McGeer and Wood, 1998).

2.5. Hormones

Hormones exert actions on every major group of tissues in the body. Behavioral and metabolic modifications are controlled by a multitude of endocrine factors. Their effects are numerous and include changes in the intermediary metabolism of carbohydrates, proteins, fats, growth and development of the tissues, changes in the permeability of membranes and the contraction or relaxation of muscles. The initiating event in the action of a hormone is its binding to its specific receptor. The combination of these two molecules results in a perturbation, such as a conformation change, that is usually followed by a chain, or cascade, of reactions that culminate in the hormone's physiological effect. The neuroendocrine system plays a central role and acts as the primary link between environmental change and physiological response in fish osmoregulatory adaptations. The system releases endocrine factors to induce the consequences of responses to overcome the external changes and/or stress (McCormick, 2001). The hormonal actions are complicated, they may be acting on the tissue directly or indirectly, stimulating or inhibiting effect, or having a dual function of which the actions can be synergistic with other factors or antagonistic to other factors.

2.5.1. Growth hormone-prolactin family

Growth hormone-prolactin family is a protein hormone family including growth hormone (GH/ somatotropin), prolactin (PRL), mammalian placental lactogen (PL/ somatomammotropin) and teleostean somatolactin (SL). A characteristic of the members of the prolactin gene family is their pleiotropic nature. GH/PRL/PL/SL gene

family evolved from a common ancestral gene approximately 850 million years ago (Rand-Weaver and Kawauchi, 1993). GH and PRL occur throughout all the major groups of vertebrates. SL appears to be confined to teleosts while PL is a mammalian hormone in the placenta.

The adenohypophysis of teleosts is divided into three main zones. They are the anterior pars distalis including rostral and proximal zones, and the posterior pars intermedia. Each adenohypophysial tropic hormone is produced by a distinct endocrine cell type in a particular region of adenohypophysis. The cells are mainly divided into two types named acidophils and basophils. Acidophils are cells with granules that can be stained by acidic dyes and basophils are cells with granules that can be stained by basic dyes. The cells that produce growth hormone and prolactin are acidophils. The acidophils that secrete GH are called somatotropes while those that secrete PRL are known as lactotropes (Norris, 1997a, b).

2.5.2. Structure of hormones

2.5.2.1. Structure of growth hormone and prolactin in fish

GH and PRL are produced in the pars distalis (Ayson *et al.*, 1993) and SL in the pars intermedia of the pituitary. All members are protein hormones of similar structure and amino acid sequence. SL is equally similar (23-28%) to both PRL and GH (Rand-Weaver and Kawauchi, 1993). All of these hormones are single globular polypeptide chains that are composed of about 200 amino acids and folded by intramolecular disulfide bridges. They have a small loop at the carboxy (COOH)-

terminus and a big loop formed by three-quarters of the remaining amino acids. They are large proteins with molecular mass of about 20 to 28 kDa. These similarities contribute to the common biological actions shared by these hormones. The hormones of this family are evolved from a common ancestral protein (Nicoll *et al.*, 1986).

The existence of PRL at extrapituitary sites has also been demonstrated in some fish. Recently, expression of the PRL gene has been demonstrated in the pituitary, liver, intestine and gonads, but not in the brain of the sea bream (*Sparus aurata*) (Santos *et al.*, 1999). The PRL gene is also expressed in the pituitary, liver, kidney, spleen, gill, muscle, gonads, and brain, but not in the intestine of goldfish (*Carassius auratus*) (Imaoka *et al.*, 2000). PRL may act in an autocrine or paracrine manner in these extrapituitary tissues.

According to the sequences of GH between higher vertebrates and fish species, GH from teleostean fish is 30-35% similar to human GH but higher sequence identity (50-70%) exists between human and more primitive fish such as Japanese eel (*Anguilla japonica*) and sturgeon (*Acipenser sturio*) (Perez-Sanchez, 2000).

The various members within the hormone family share similar biological activities. PRL exhibits GH-like activity whereas GH has PRL-like actions. Such common properties of these hormones are due to the similarities in their amino acid sequences, common sites of intramolecular disulphide bridges and three-dimentional structure of the molecule. PRLs have been isolated and their amino acid sequences determined in most of the major groups of the vertebrates (Specker *et al.*, 1985; Yamauchi *et al.*,1988) . There are fewer amino acid residues in fish than other

vertebrates. There are greater differences showing in chemical structure among the PRLs than the GHs of vertebrates. Especially notable are the PRLs in teleost fish, which, apart from being smaller, only contain two disulfide bridges instead of three as seen in most other vertebrates. Compared with mammalian PRL, all teleost PRLs lack the N-terminal disulfide bond due to the absence of 12-14 amino acids at the N-terminus (Rand-Weaver and Kawauchi, 1993). It is possible that the loss of the N-terminal disulfide bridge in teleosts, may be related to the central role of PRL as an osmoregulatory hormone in these organisms.

Two prolactin (PRL) forms have been shown in some teleosts, for example, chum salmon (*Oncorhynchus keta*), common carp (*Cyprinus carpio*), Japanese eel (*Anguilla japonica*), Mozambique tilapia (*Oreochromis mossambicus*) and Nile tilapia (*Oreochromis niloticus*) (Suzuki *et al.*, 1991). The salmon, carp, and eel PRLs are highly homologous, whereas in some teleosts such as in tilapia and cichlids, the two PRLs share only 69% amino acids identity (Yamauchi *et al.*, 1988; Rentier-Delrue *et al.*, 1989; Specker *et al.*, 1993). The PRLs are classified as long (188 amino acids; PRL_{188}) and short (177 amino acids; PRL_{177}) forms (Yamaguchi *et al.*, 1988). Both PRLs appear to be equally efficient at preventing the loss of plasma Na^+ and Cl^- and at maintaining the plasma osmolality and whole-animal transepithelial potential of hypophysectomized *Oreochromis mossambicus* in FW (Young *et al.*, 1988). The effects of PRL_{177} and PRL_{188} may differ depending on the environmental salinity and the species being investigated. PRL_{177} acts to decrease water permeability while PRL_{188} is primarily involved in the regulation of plasma Na^+ and Cl^- when fish are adapted to hyperosmotic environments (Auperin *et al.*, 1994). PRL_{188} binding was greater than PRL_{177} in all tissues examined including gill, kidney, gut, skin and liver.

The highest levels of binding were observed in the kidney followed by the gill. Differences in the biological activites of PRL_{188} and PRL_{177} are not due to the independent regulation of different cell types or secretion granules (Specker *et al.*, 1993).

The effects of PRL on target tissues are mediated by a receptor. PRL-binding proteins may serve to protect circulating PRL from degradation, thus providing a "ready-reserve" of the hormone to allow for rapid responses to environmental changes such as salinity challenges. The prolactin receptor (PRLR) is a member of the class 1 cytokine receptor superfamily that includes receptors for GH, erythropoetin, leptin, and the interleukins. All of these members are single-pass, transmembrane chains and are classified as class 1 cytokine receptors based on the presence of several highly conserved, functionally important amino acid sequences in their extracellular and intracellular domains (Bole-Feysot *et al.*, 1998). One PRL receptor was present regardless of the environmental salinity, but transfer from freshwater to brackish water increased both binding of PRL_{188} and PRL_{177} to receptors, the receptor affinity for PRL_{188} and the number of PRLRs. PRLs may exert inhibitory control over their own receptor. An increase in environmental salinity resulted in a decrease in the expression of the PRLR. Thus, it appears that PRLR mRNA expression is upregulated by the higher circulating PRL levels present in FW (Ayson *et al.*, 1993; Auperin *et al.*, 1994). However, the number of PRLRs and specific binding of tPRL increased following transfer of *Oreochromis niloticus* to a hyperosmotic environment, suggesting a down-regulation of the PRLR protein in the presence of elevated PRL levels in freshwater, perhaps due to an increase in internalization and degradation of the PRLR under these conditions. The mechanism

of ligand binding and receptor activation is also conserved between fish and mammals. PRLRs were localized to the chloride cells of the gill and the mucosal layer of the intestine, consistant with the role of PRL and these cells in osmoregulation (Sandra *et al.,* 1995, 2000).

Comparing the PRL and GH receptors, in tilapia, specific radioimmunoassays (RIAs) for the pair of tilapia PRL (tPRLs) and GH (tGH) were developed using antisera raised in rabbits. Anti-tPRL177 did not cross-react with tPRL188 and tGH. Anti-tPRL188 did not cross-react with tPRL177 and showed slight cross-reaction (3.1%) with tGH. Anti-tGH showed negligible cross-reactions with tPRL177 (0.4%) and tPRL188 (1.6%). Ovine PRL probably could not distinguish between fish GH and PRL receptors and therefore was not suitable for the molecular and physiological characterization of fish PRL receptors (Prunet and Auperin, 1994).

2.5.2.2. Structure of insulin-like growth factors in fish

Insulin and insulin-like growth factor-I (IGF-I or somatomedin C) belong to the insulin superfamily. Insulin gene family in vertebrates includes two closely related hormones named insulin-like growth factor (IGF-I and -II). They are structurally related to insulin, although they are expressed ubiquitously. In fact, IGFs have both growth-promoting effects and insulin-like activity. IGF-I is the form of IGF produced in adults and IGF-II is a fetal growth factor (Bentley, 1998).

IGF-I is a polypeptide composed of 70 amino acids and is highly conserved in amino acid sequence among vertebrate. IGF-I of human differs from those of

domestic fowl, toad and Atlantic fish by 8, 11 and 14 amino acids respectively (Bentley, 1998). Both IGF-I and IGF-II bind to specific high-affinity carrier proteins, insulin-like growth factor binding proteins (IGFBPs) in blood plasma to form a complex. IGFBPs can be found in all vertebrates including teleosts. IGFBPs prolong the half-life of IGFs and control the activities of IGFs by preventing receptor binding (Clemmons, 1992; Bach and Rechler, 1995). The biological functions of the IGFs are mediated by different transmembrane receptors that include the insulin, IGF-I and IGF-II receptors. Receptors for IGF-I and insulin belong to the same family. Both ligands and receptors are structurally related and have overlapping functions (Massague and Czech, 1982). Each IGF-I receptor is a heterotetramer that consists of two α-subunits and two β-subunits linked by disulfide bridges and has intrinsic tyrosine kinase activity of which the IGF-II receptor lacks. The α-subunit is the extracellular hormone (ligand) binding recognition site whereas the β-subunit contains extracellular, transmembrane and the cytoplasmic or intracellular tyrosine kinase domains (Soos and Siddle, 1989). Binding of IGF-I to the extracellular recognition site results in autophosphorylation of the intracellular tyrosine kinase domain (White *et al.*, 1987). The tyrosine kinase is activated and is able to phosphorylate tyrosine residues of insulin receptor substrate (IRS) family of proteins that mediate intracellular signaling by IGF-I and insulin (Mommsen, 1998).

2.5.2.3. Structure of cortisol in fish

Cortisol is the main adrenocorticosteroids in teleost fish. It is a 21-C steroid produced by the interrenal tissue. The interrenal tissue of teleosts is homologous to the adrenal cortex of mammals (Wendelaar Bonga, 1997).

2.5.3. Regulation of hormones

2.5.3.1. Regulation of growth hormone in fish

Hypothalamic control of growth hormone (GH) secretion is regulated by complex interactions at hypothalamic and pituitary sites with numerous stimulatory and inhibitory neurohormones acting on the somatotrophs seasonally. Growth hormone transcription and secretion are primarily controlled by two hypothalamic factors. They are stimulated by growth hormone-releasing hormone (GHRH) or factor (GRF) and inhibited by somatostatin (somatotropin release-inhibiting hormone / SRIH or factor / SRIF). SRIH may alter GH synthesis by modulation of translation (Melanded, 1998). The presence of specific GH-binding sites on the central nervous system of rainbow trout has been reported suggesting that in fish GH feedback at the hypothalamic level to alter GHRH and SRIH release (Perez-Sanchez *et al.*, 1991). There are several metabolic effectors that interact at the central level and contribute to establish the control of pituitary GH release. Two native gonadotropin-releasing hormones (GnRH and GnRH-II) stimulate GH release via activation of phospholipase C, protein kinase C, Ca^{2+} entry through voltage-sensitive channels and calmodulin (Melanded, 1998). Activation of adenylyl cyclase and protein kinase C by forskolin and synthetic phorbol esters triggers GH release. Chang *et al.* (1991) established that inhibitors of protein kinase C [1-(5-isoquinolininesulfonyl)-2-methylpiperazin dihydrochloride and calcium channel blockers (verapamil) exert an inhibitory effect on GH release in goldfish. In addition, GH secretion is also stimulated by a number of neuroendocrine factors, including growth hormone-releasing factor (GRF), dopamine (DA), neuropeptide Y (NPY), thyrotropin-releasing hormone (TRH), cholecystokinin

(CCK), bombesin (BBS) and activin. Somatostatin is the primary inhibitor of basal and stimulated GH secretion. Norepinephrine and serotonin also have inhibitory actions on GH. Sex steroids, in particular estradiol, influence the responsiveness of the somatotrophs to neuroendocrine factors, in other words, influence GH secretion. The basic mechanisms of GH synthesis and release are conserved in vertebrate evolution.

T_3 increases GH mRNA levels (in tilapia and common carp) but insulin-like growth factor-I (IGF-I) reduces GH transcript levels as well as inhibiting GH release. Physiological levels of circulating cortisol would be necessary to maintain normal pituitary GH secretion (Theill and Karin, 1993). In contrast, when plasma cortisol concentration increases above the normal range, there is an increase in hypothalamic SRIH which overrides stimulatory effects (Giustina and Wehrenberg, 1992). This can be an explanation why handling and confinement stress induce a rapid and consistent decrease of plasma GH levels in several fish species (Pickering *et al.*, 1991; Auperin *et al.*, 1997). With increasing the age, a progressive and significant decreasing trend of pituitary GH gene expression was observed (Marti-Palanca *et al.* 1996). Decreasing water temperature, plasma GH levels also decrease (Perez-Sanchez and Le Bail, 1999). Increasing daylength during the spring period stimulates pituitary GH synthesis and release in seabream, goldfish and smoltifying salmon (Perez-Sanchez *et al.*, 1994; Marchant and Peter, 1986; Dickhoff *et al.*, 1997).

Both in juvenile and adult fish, the liver is the most important target for the direct action of GH (Hirano, 1991). Liver cells activated by GH produce a fish insulin-like growth factor (IGF, probably IGF-I; Drakenberg *et al.* 1989). In seabream

larvae the greater concentration of GH-binding sites occurs in the head region suggesting that at this early stage of development, GH exerts a direct action on growing tissues rather than a systemic one mediated by hepatic IGF-I. The GH-liver axis is a sensitive marker of growth performance in later stages of growth (Perez-Sanchez, 2000). Fasting and malnourished fish show a decrease in hepatic GH-binding and circulating IGF-I, which increases pituitary GH release due to a lack of negative feedback inhibition (Fig. 2.3). Perez-Sanchez *et al.* (1992) established that human IGF-I inhibited GH release on *in vitro* cultures of rainbow trout pituitary cells in a dose dependent manner and IGF-I also stimulates SRIH release and inhibits GHRH release. Together with the observation of Cao *et al.* (1989) that GH treatment increases IGF-I activity, we can conclude that IGFs participate in the negative feedback loop of fish GH release. Specific GH-binding sites have also been detected in several extrahepatic tissues such as testis, muscle, brain, gills, adipose tissue, spleen, cartilage (Perez-Sanchez *et al.*, 1991; Yao *et al.*, 1991).

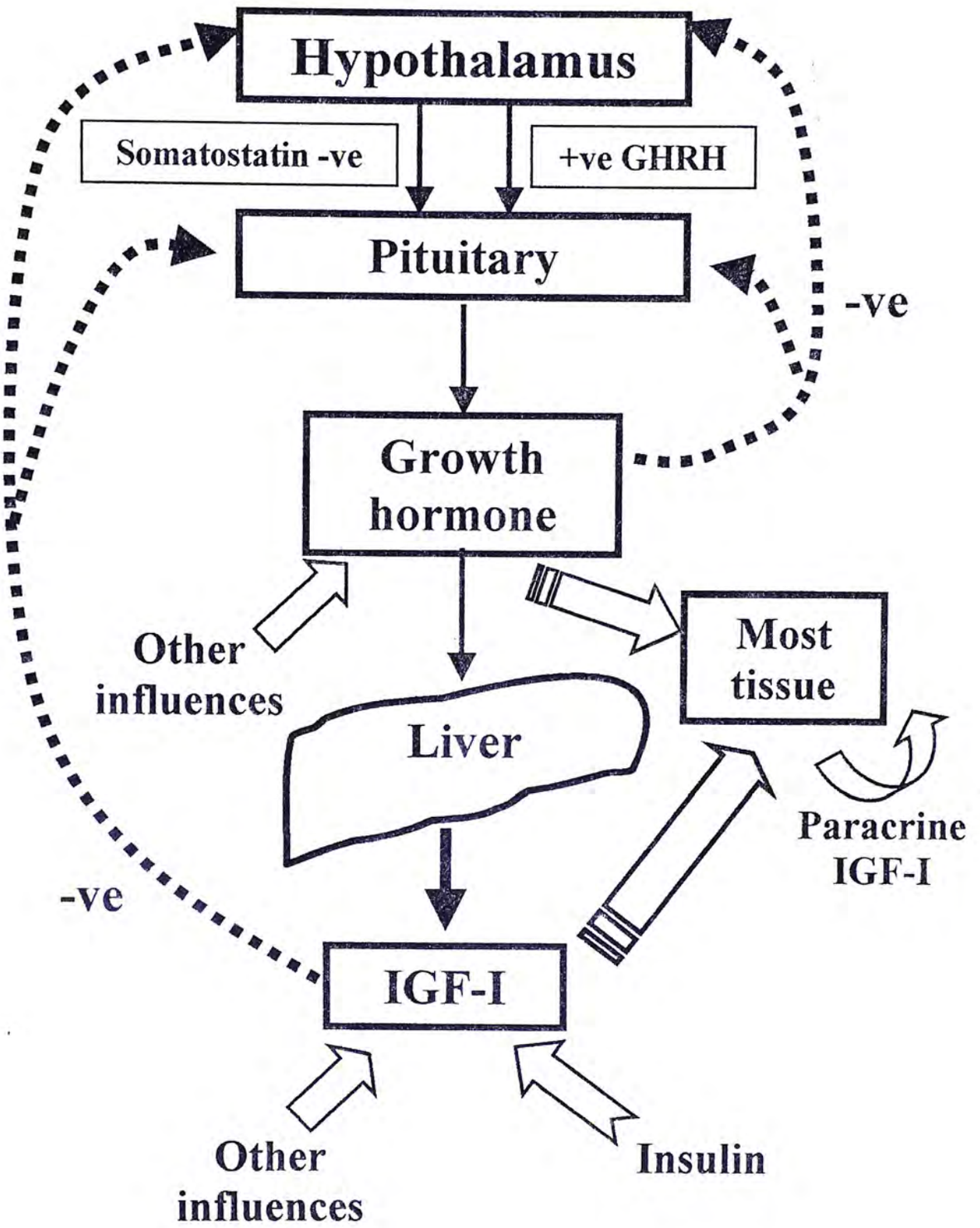


Fig. 2.3 The growth hormone/insulin-like growth factor I axis

2.5.3.2. Regulation of prolactin in fish

The major control of prolactin release is inhibitory from the hypothalamus. Prolactin release is inhibited by dopamine (DA), a catecholamine which can stimulate the release of growth hormone in goldfish (Wong *et al.*, 1992). Thyroid-stimulating hormone (TRH) from hypothalamus can stimulate prolactin release but it can also stimulate thyroid-stimulating hormone (TSH; thyrotropin) release.

In teleost, some studies conjectured that changes in osmolality or sodium levels but not alteration of plasma calcium concentration seem to be more critical for the regulation of PRL release. These processes may be reflected by changes in the storage of PRL in the adenohypophysis. During adaptation to freshwater, pituitary and plasma PRL levels increase. This shows that the synthesis and release PRL is influenced by the salinity of the external medium. PRL gene expression is decreased after seawater transfer regardless of seawater adaptability (Yada and Hirano, 1992; Yada *et al.*, 1994). Both the plasma and pituitary levels of the two PRLs (PRL_{177} and PRL_{188}) decreased significantly when transfer from fresh water to seawater, but both PRLs increased after transfer from seawater to fresh water in tilapia *Oreochromis mossambicus* and *Oreochromis niloticus* and the synthesis, secretion and metabolic clearance of the two tPRLs could be independently regulated in the same cells (Ayson *et al.*, 1993).

2.5.3.3. Regulation of insulin-like growth factor-I in fish

IGF-I is produced in multiple tissues but predominately is secreted in the liver and delivered to the circulation. The most important secretogogue is GH and plasma IGF-I regulates GH release through negative feedback mechanism at the levels of hypothalamus and pituitary. Fasting abolished the stimulatory action of GH on hepatic IGF-I synthesis and release (Duan 1998).

2.5.3.4. Regulation of cortisol in fish

Stressors, either acute or chronic, increase plasma cortisol levels by activation of the hypothalamo-pituitary-interrenal (HPI) axis. Under stressful conditions, hypothalamic corticotropin-releasing hormone (CRH) stimulates the release of a tropic hormone, adrenocorticotropic hormone (ACTH or corticotropin), from corticotropes in rostral pars distalis of adenohypophysis, which in turn, stimulates the secretion of cortisol from the interrenal tissue. Cortisol helps the animal to cope with stress by mobilization of energy reserves and maintenance of ionic balance (Hazon and Balment, 1998).

2.5.4. Functions of hormones

2.5.4.1. Functions of growth hormone in fish

GH is an adenohypophysial hormone, as the name 'growth hormone' suggests the ancestral role for GH is growth, acting as a growth-promoting hormone. It has

been implicated in promotion of growth in mammals. In fishes, GH has various functions such as growth stimulation, regulation of carbohydrate, lipid and protein metabolism and affecting reproductive, immune, endocrine, neuroendocrine and neural functions, and the most important effect in fish is osmoregulation. It also appears to be involved in stress adaptation and reproduction as all these processes are strongly interdependent (Le Bail *et al.*, 1991).

GH has a mitogenic effect and it is more persistent than IGF-I and the combination of GH and IGF-I can produce synergistic effects in somatogenic action (Fielder *et al.*, 1996). Growth in fish is regulated largely by the brain neuroendocrine-GH-IGF-I axis. High doses of homologous GH stimulated growth, as was indicated by increases in length and mass (Rentier-Delrue *et al.*, 1989). Greater concentration of GH-binding sites occurs in the head region of fish larvae which exhibits a fast growth, suggesting GH exerts a direct action on growing tissues rather than through a systemic effect mediated by hepatic IGF-I at this early stage of development (Funkenstein and Albuly, 1996). However, the GH-liver axis is a sensitive marker of growth performance at the later stages (Perez-Sanchez and Le Bail, 1999). GH also plays important role in controlling differentiation, hypertrophy and metabolism of muscle (Dauncey, 1995). The growth-enhancing property of growth hormone has been attributed to increase feed consumption and improved feed efficiency (Markert *et al.*, 1977).

GH is needed for maintaining diet composition and feeding strategies. The brain peptide systems regulating food intake are linked to the brain neuroendocrine regulation of GH secretion. Following a meal, fish characteristically show a short-

term increase in serum GH concentrations to inhibit food uptake, and then a decrease in serum GH concentrations to below premeal levels (Peng and Peter, 1997). GH also promotes fat mobilization by increasing lipolysis and free fatty acid oxidation and decreasing lipogenesis. Implantation of ovine growth hormone (bGH) in juvenile coho salmon, *Oncorhynchus kisutch* in both early and late stages of smoltification, stimulated lipid mobilization by increased lipolytic enzyme (triacylglycerol lipase) activity thus enhancing lipolysis (Sheridan, 1986). During fasting or malnutrition, increase in circulating GH levels has been demonstrated in several fish species (Perez-Sanchez *et al.,* 1995). GH replacement restored lipase activity of hypophysectomized smolts. When energy is largely available, GH protects adipose tissue and other organs and tissues from excessive lipid deposition (Company *et al.,* 1999). These findings suggest that under conditions of both energy deficit and energy excess, GH is useful to increase energy availability during fasting and energy restriction but at the same time, it prevents further weight gain and adiposity in overfed fish. GH is also protein anabolic because it stimulates uptake of amino acids and protein synthesis and inhibits nitrogen excretion, especially in skeletal muscle and bone. GH is also important in carbohydrate metabolism and acts as a diabetogenic hormone. However, this is related to its actions on lipid and protein metabolism. It is because blood glucose concentrations are usually increased (hyperglycemia) as fatty acid oxidation, glycolysis and glycogenesis are inhibited (Dauncey, 1995; Bentley, 1998).

The roles of growth hormone in metabolism and osmoregulation are very important in sexual maturation during parr-smolt transformation in salmonids (Bjornsson, 1997). The less energy reserved for metabolism and osmoregulation, the more energy is available for repoduction. GH is also required for normal reproductive

functioning, directly influencing gonadal function in fish. For instance, ovarian growth in Coho salmon is stimulated by the administration of bovine GH *in vivo* (Higgs, 1977). Fish GH replacement therapy increased testicular weight, plasma concentration of testosterone and testosterone synthesis in hypophysectomized adult male killifish (Singh, 1988).

GH receptors are present in erythroid, lymphoid and myeloid cells and GH is able to exert a direct effect on the haemopoietic tissues (Calduch-Giner *et al.*, 1995) indicating GH also important in immune functions in a number of fish species by acting as a phagocytic-activating factor (Narnaware and Woo, 1999; Perez-Sanchez, 2000). The proliferation action of GH on *in vitro* leukocyte cultures of both sea bream and chum salmon provides the evidence for a role of GH as a fish erythropoietic, myelopoietic and lymphopoietic growth factor.

GH has specific calcitropic effects in freshwater tilapia. In GH-treated fish, the net efflux of calcium was lower than in the controls, however, the calcium influx via the gills was not affected. Calcium taken up from the water was more efficiently stored in the body. GH increased the total body calcium pool without affecting bone or scale calcium density (Flik, 1993).

Furthermore, subsequent research showed that GH exerts considerable effects on osmoregulation. It is generally accepted that GH is involved in seawater adaptation by stimulating ion extrusion. GH was able to reverse partially the effects of hypophysectomy on coho salmon (*Oncorhynchus kisutch*) as judged by improved seawater (SW) tolerance after long-term treatment. It reduces the magnitude of

physiological changes induced by exposure to high salinity during transfer from brackish water to seawater in many salmonid species as judged by lower plasma sodium, osmolality such as changes in plasma Na^+, Cl^-, total Mg concentration and muscle moisture content (Sakamoto *et al.*, 1990, 1991, 1993; Seidelin *et al.*, 1999). Long term treatment with GH increased in gill Na^+-K^+-ATPase density (Leena and Oommen, 2000), activity *in vitro* (McCormick and Bern, 1989) and *in vivo* (Richman and Zaugg, 1987a,b; Xu *et al.*, 1997; Seidelin *et al.*, 1999) as well as gill sodium efflux into seawater. In addition, some studies also reported the elevation in chloride cell numbers, apical area, fractional area (Sakamoto *et al.*, 1993; Xu *et al.*, 1997; Kelly *et al.*, 1999a,b) or chloride cell proliferation (Perry, 1998) or expression of Na^+-K^+-ATPase α-subunit (Madsen *et al.*, 1995; Sakamoto *et al.*, 1997; Mancera and McCormick, 1998) and drinking rate or all the parameters (Bolton *et al.*, 1987; Madsen, 1990a,b; Sakamoto *et al.*, 1993; Boeuf *et al.*, 1994; Fuentes *et al.*, 1997) after exposure to seawater. GH reduced renal Na^+-K^+-ATPase activity but did not change intestinal Na^+-K^+-ATPase activity in 33‰ (SW) silver seabream *Sparus sarba* (Kelly *et al.*, 1999b). The $Na^+/K^+/Cl^-$ cotransporter in gill chloride cells of Atlantic salmon was also upregulated by GH (Pelis and McCormick, 2001).

Treatment with GH increased standard oxygen consumption in rainbow trout (*Oncorhynchus mykiss*) by 18% in FW and a further 12% after transferred to SW (Seddiki *et al.*, 1995), the elevated rate of oxygen uptake apparently led to improved seawater adaptability. GH treatment induced an increase in the size and number of α-chloride cells and accessory cells (both thought to be involved in salt secretion), however, GH treatment decreased the number of the β- chloride cells (putative ion uptake cells), which are thought to be specific to freshwater life (Shikano and Fujio,

1999). Such functional and ultrastructural modifications suggested that GH stimulated the differentiation of freshwater chloride cells toward a seawater type. Another study also revealed the same phenomenon, GH had no effect on brown trout (*Salmo trutta*) gill primary filaments Na^+-K^+-ATPase immunoreactive cell density but reduction of these on the secondary lamellae were observed (Seidelin *et al.*, 1999). It seems that there was a redistribution of Na^+-K^+-ATPase immunoreactive cells away from the secondary lamella onto the primary filament of FW-acclimated trout.

At the opposite end of the salinity spectrum, despite the clear ''seawater-adapting effects'' of GH in many salmonids, recent evidence suggests that GH in general seems also to enhance hyperoosmoregulatory ability of fish. GH-induced alterations in the branchial epithelium of freshwater-acclimated fish such as in salmonids had greater salinity tolerance than controls. They had greater gill Na^+-K^+-ATPase activity and elevation in branchial Na^+ uptake which occurred via an increased presence of electrogenic H^+-ATPase (Perry and Fryer, 1997). GH reduced renal Na^+-K^+-ATPase activity but elevated activity in the anterior regions (esophagus and stomach), and at the same time stimulated chloride cell fractional area and exposure numbers in 6‰-adapted (hypoosmotic condition) silver seabream *Sparus sarba* (Kelly *et al.*, 1999c). In freshwater tilapia, the chloride cell density in the opercular epithelium almost doubled with no effect on plasma osmolality after GH treatment (Flik, 1993).

2.5.4.2. Functions of prolactin in fish

Prolactin has been shown to influence immune functions in a number of fish species. The numerous functions of PRL fall into several categories: reproduction, endocrinology, metabolism, osmoregulation, growth, development, immunoregulation and protection (Bern, 1983; Brown and Brown, 1987; Sakamoto *et al.*, 1991; Bole-Feysot *et al.*, 1998; Narnaware *et al.*, 1998). It has been demonstrated that PRL binds to PRL receptors that are present in the osmoregulatory organs such as gill, kidney, intestine, urinary bladder and skin, indicating that these are physiological targets for the direct action of PRL (Dauder *et al.*, 1990). There are marked species differences in the actions of PRL on target organs.

The effects of PRL on fish osmoregulation have been the focal point of a wide variety of studies. Fish that reside in a freshwater environment face two primary challenges: preventing the loss of ions to the external hypoosmotic environment and preventing the influx of water. Death in freshwater following hypophysectomy was found in several species which was accompanied by a considerable loss of sodium. Pickford and Philips (1959) first discovered that PRL enabled hypophysectomized killifish (*Fundulus heteroclitus*) to survive in freshwater. No other hormones were found to have this effect. PRL plays a central role in the control of these activities during the adaptation of fish to freshwater, as evidenced by its ability to (1) increase plasma ion concentrations (primarily Na^+ and Cl^-) by reducing the rate of sodium chloride secretion from the branchial chloride-secreting cells, for example, reduced activity of the chloride cells or inhibition of ion transporters (Clarke and Bern, 1980; Hirano, 1986; Pisam *et al.*, 1993; Yada and Ito, 1999) and (2) prevent both the loss of

ions (especially Na^+ and Cl^-) by decreasing the permeability of the cell membranes of osmoregulatory organs (gill, kidney, intestine, urinary bladder, skin) to water (Hirano, 1986; Pisam *et al.,* 1993; Yada and Ito, 1999; Eckert *et al,* 2001). In diadromous and euryhaline fish, circulating PRL levels decline when fish are transferred from freshwater to seawater (Hasegawa *et al.*, 1987; Yada and Hirano, 1992; Yada *et al.*, 1994; Auperin *et al.*, 1995; Morgan *et al.,* 1997) or increase when fish are transferred from seawater to freshwater (Hasegawa *et al.*, 1987; Yamauchi *et al.*, 1991). PRL showed sodium retaining activity by preventing a decrease in plasma sodium levels after acid exposure (Yada and Ito, 1999). A significant reduction in PRL cell activity occurred after transfer to 80% SW, but not after transfer to Ca-rich FW (Kakizawa *et al.*, 1993).

Numerous studies have reported the inhibitory effects of PRL on a key ionomotive enzyme, Na^+-K^+-ATPase in both seawater and 6‰ adapted fish (McCormick, 1995; Sakamoto *et al.,* 1997; Kelly *et al.,* 1999c) and thus functions by preventing the loss of ions to the external hypoosmotic environment. There is no clear consensus with respect to the effects of PRL on gill Na^+-K^+-ATPase activity. However, some studies showed that PRL cause no significant effect in the abundance of alpha-subunit gill Na^+-K^+-ATPase mRNA and activity (Madsen *et al.*, 1995; Young *et al.,* 1988; Yada and Ito, 1999; Eckert *et al.,* 2001) or increases on gill Na^+-K^+-ATPase activity (Boeuf *et al.,* 1994; Leena and Oommen, 2000). These discrepancies may be related to the use of heterologous PRLs where these PRLs may bind to both GH and PRL receptors, or these may be due to species variability or developmental differences (McCormick, 1995). Looking at the effects of ovine PRL on the expression of the catalytic α-subunit and the β-subunit of Na^+-K^+-ATPase in

fish, PRL caused a significant reduction in gill Na^+-K^+-ATPase α-subunit, but not in β-subunit mRNA levels in silver seabream (*Sparus sarba*) (Deane *et al.*, 1999), however, α-subunit mRNA levels were not affected by PRL in the brown trout (*Salmo trutta*) (Seidelin and Madsen, 1999). To fully elucidate the effects of PRL on gill Na^+-K^+-ATPase, studies should be carried out on different species of fish using homologous hormones.

In 6‰ acclimated saline-administrated *Sparus sarba*, elevated branchial chloride cell numbers and exposure indices were observed, all of which were markedly reduced by oPRL. CC numbers and morphometrics were unaffected by oPRL in seawater fish. but oPRL had no effect on renal Na^+-K^+-ATPase activity. Treatment with oPRL elevated Na^+-K^+-ATPase activity throughout the gastrointestinal tract of seawater fish and in the anterior reaches of 6‰ adapted fish (Kelly *et al.*, 1999c). PRL exerts a hypercalcaemic action in addition to its well-established hypernatraemic effect. Treatment of seawater fish with PRL resulted in hyperchloremia, stimulating Ca^+ uptake via the branchial Ca^{2+} pump (Flik *et al.*, 1989) whereas in another study, prolactin appears to have no direct role in the calcium regulation (Pandey, 1990).

Preventing the influx of water, PRL stimulates proliferation of epidermal mucocytes of the gill to increase mucus secretion impeding the passage of molecules in and out of gill cells (Bentley, 1998). PRL decreases the osmotic permeability of the gills (Brown and Brown, 1987). PRL acts on the mucous cells that may contribute to the regulation of ion and water balance in FW by. PRL is also able to prime trout phagocytes for *in vitro* superoxide anion release, which suggests that it is involved in

immune response in fish (Sakai *et al.*, 1996). Similar stimulation of the immune response by prolactin in the phagocytes in seabream has been observed (Narnaware *et al.*, 1998).

Hypo-osmoregulatory ability of seawater-transferred brown trout (*Salmo trutta*) was impaired by PRL injection as judged by changes in plasma osmolality, Na^+, Cl^-, total Mg concentration and muscle water content. PRL had no effect on both primary filaments and secondary lamellar Na^+-K^+-ATPase immunoreactive cell density in brown trout (*Salmo trutta*) gills (Seidelin *et al.*, 1999).

PRL appears to have a marked effect on the morphology, distribution and number of chloride cell. PRL has been reported to either reduce or have no effect on chloride cell numbers and to decrease chloride cell in the average cross-sectional area and height of chloride cells (Herndon *et al.*, 1991). However, the effects of PRL may vary with environmental salinity so that the number and size of chloride cell in *Sparus sarba* were reduced by PRL in a hypoosmotic environment, but not in SW (Kelly *et al.* 1999c). PRL injections caused a decrease in α cell size and a dedifferentiation to the freshwater α cell morphology.

PRL strongly stimulated lipid mobilization in all regions (liver, dark muscle, mesenteric fat) in salmon parr. Lipase activity increased leading to either decrease in total lipid concentration or in total tissue mass (mesenteric fat) and tissue triacylglycerol content. Smolts (characteristically possessing elevated liver lipase activity) that were hypophysectomized exhibited low levels of liver lipase activity. These results indicate that PRL stimulates lipid mobilization in developing salmon by

enhancement of lipolysis and suggest that PRL contribute to smoltification-associated lipid depletion (Sheridan, 1986).

Comparing the function of different PRLs, both PRL_{177} and PRL_{188} increased plasma osmolality in 100% seawater and reduced gill Na^+-K^+-ATPase activity, the effects induced by PRL_{188} being more significant than those by PRL_{177}. Lower ratio of PRL_{177}: PRL_{188} was apparent in freshwater compared with seawater-adapted tilapia (Borski *et al.*, 1992). Similarly, the transfer of *Oreochromis niloticus* from freshwater to either brackish or seawater resulted in a decrease in plasma and pituitary levels of the two tPRLs and an increase in the ratio of PRL_{177}: PRL_{188} (Auperin *et al.*, 1994; Morgan *et al.*, 1997) suggesting that PRL_{188} is more sensitive to freshwater adaptation.

The somatotropic actions of PRLs have also been examined. Injections of tilapia PRL_{188} but not PRL_{177} were able to promote growth as measured by an increase in length and weight of the fish (Specker *et al.*, 1985). Conversely, tilapia PRL_{177} but not PRL_{188} had somatotropic activity (Shepherd *et al.*, 1997).

Prolactin treatment also resulted in osmoregulatory derangements in the intact fish. PRL has been reported to induce reductions in passive branchial Na^+ loss, Na^+-K^+-ATPase activity, chloride cell numbers and chloride cell exposure to the external environment and osmotic water permeability (Kelly *et al.*, 1999c, Madsen and Bern, 1992; Hirano, 1986). This is of particular relevance to the present study since Kelly *et al.* (1999c) reported similar roles of PRL in the silver seabream *Sparus sarba*.

2.5.4.3. Functions of insulin-like growth factor-I in fish

Biological function of IGF-I has been highly conserved among vertebrates. It was also called "sulfation factor" or "somatomedin C" because of the incorporation of sulfate into cartilage leading to growth. It is a mitogenic polypeptide that plays an essential role in the regulation of development and somatic growth of vertebrates, mainly by mediating GH actions (Duan and Hirano, 1990; Sakamoto and Hirano, 1991; Gray and Kelley, 1991; McCormick *et al.* 1992).

IGF-I has been demonstrated in both of the gonads, testis and the ovary. This implies a role in spermatogonial proliferation and in oocyte maturation. IGF-I is able to stimulate both germinal cells and somatic cells production (Fostier *et al.*, 1994).

Plasma IGF-I (McCormick *et al.*, 1991), and hepatic, branchial and renal levels of IGF-I mRNA (Sakamoto *et al.*, 1995) increase during smolting of coho salmon and following seawater transfer. IGF-I increased salinity tolerance as evidenced by decreasing in plasma osmolality in seawater-transferred brown trout (*Salmo trutta*) administered with IGF-I. There was no significant effect on plasma total Mg concentration and muscle water content. GH or IGF-I injected brown trout (*Salmo trutta*), exhibited stimulated branchial Na^+-K^+-ATPase α-subunit mRNA and activity but both these variables were unaffected in kidney and posterior intestine (Madsen *et al.* 1995; Seidelin *et al.*, 1999). Na^+-K^+-ATPase activity decreased in pyloric caeca and middle intestine by IGF-I (Seidelin *et al.*, 1999). The stimulation can be explained by the increased expression within Na^+-K^+-ATPase immunoreactive (NKIR) cells in individual primary filaments. rhIGF-I increased NKIR cell density in

gill primary filaments but decreased secondary lamellar NKIR cell density in brown trout (*Salmo trutta*).

2.5.4.4. Functions of cortisol in fish

Cortisol, the major corticosteroid in the majority of teleost fish species, plays a dual role in contributing to the regulation of both carbohydrate metabolism as a glucocorticoid and osmoregulation as a mineralocorticoid (Bern and Madsen, 1992; Wendelaar Bonga *et al.*, 1997; Mommsen *et al.*, 1999). The possible role of cortisol in teleost osmoregulation has been studied by observing the effects of interrenalectomy in eels. Interrenalectomy in freshwater European eels results in a loss of sodium ions that can be corrected by the injection of small amounts of cortisol, affecting both renal and branchial functions (Chan *et al.*, 1967). In the seawater-adapted eels, this surgery results in an excessive accumulation of sodium (Mayer *et al.*, 1967). Cortisol was also able to reverse partially the effects of hypophysectomy on coho salmon (*Oncorhynchus kisutch*) as judged by improved freshwater and seawater tolerance after long-term treatment, however, it cannot significantly restore gill Na^+, Na^+-K^+-ATPase activity (Richman *et al.*, 1987).

Most of the species (e.g. eels), in correlation with the increase in plasma osmolality, increased cortisol circulating levels and metabolic clearance after exposure to seawater (Perry and Wood, 1985; Perry and Laurent, 1989; Flik *et al.*, 1989; Wood & Marshall, 1994), the elevation may act primarily to promote seawater adaptating mechanisms. Not all teleosts respond to cortisol in this manner. Atlantic salmon smolts display a drop in plasma cortisol concentrations following transfer to seawater (Langhorne and Simpson, 1986). Plasma cortisol is a primary indicator of

stress. Elevation of plasma cortisol is a reliable and the most widely used indicator of stress in fish (Wendelaar Bonga, 1997). The secretion of cortisol increased under stress, such as under net confinement (Vijayan *et al.*, 1997; Nolan *et al.*, 1999), infection (Mesa *et al.*, 1999) and exposure to acid. Cortisol treatment also increased the ability of acid-exposed fish by stimulating gill Na^+-K^+-ATPase activity to maintain plasma sodium levels in medaka (*Oryzias latipes*) (Yada and Ito, 1999).

There is great diversity among teleost fishes, and individual species and effector organs will differ in the relative importance of cortisol in controlling ion uptake and secretory mechanisms. It is thought to be a seawater-adapting hormone because it promotes salt excretion in hypo-osmoregulating fish by increasing Na^+-K^+-ATPase expression within osmoregulatory organs including the gill, intestine and kidney such as the abundance of Na^+-K^+-ATPase α-subunit mRNA and activity and/or promote the proliferation, differentiation and the development of chloride secretory cells within the gill epithelium relative to untreated control fish (Richman and Zaugg, 1987a,b; McCormick and Bern, 1989; Madsen, 1990; McCormick, 1990, 1995; Hwang and Wu, 1993; Madsen *et al.*, 1995; Marshall *et al.*, 1999; Seidelin *et al.*, 2000; Kelly and Wood, 2001).

Cortisol administration caused a significant elevation in the abundance of Na^+-K^+-ATPase α-mRNA but the level of Na^+-K^+-ATPase β-mRNA, Na^+-K^+-ATPase activity, branchial chloride cell number, exposure and apical area were unchanged, indicating that cortisol treatment could not induce Na^+-K^+-ATPase translation in hypo-osmoregulating *Sparus sarba* (Deane *et al.*, 2000).

Cortisol may be also involved in the early phase of seawater adaptation in developing larvae. Tilapia embryos or larvae treated with cortisol and then transferred to seawater (SW) showed reduced cumulative larval mortality in seawater compared with controls. It is suggested that cortisol treatment significantly diminished the amplitude of increase in larval body Na^+ content by increasing the Na^+-K^+-ATPase of yolk-sac epithelia after transfer to SW (Guggino, 1980) and absorption of Na^+ and Ca^{2+} in freshwater in embryos and larvae have been demonstrated via chloride cells (McWilliams and Shephard, 1989; Hwang *et al.*, 1994, 1996). Moreover, the mechanism of water balance was also found to be developed at early developmental stages in teleosts (Guggino, 1980; Tytler and Blaxter, 1988).

In some species (e.g. salmonids, rainbow trout), cortisol may have the capacity to stimultaneously increase ion (Na^+, Ca^{2+}, Cl^-) uptake by increasing the number of chloride cells (Richman *et al.*, 1987; Perry and Laurent, 1989; Laurent and Perry, 1990; Flik *et al.*, 1989; Laurent *et al.*, 1994; McCormick, 2001; Dang *et al.*, 2000). Cortisol treatment significantly increased the ion regulatory capacity of the gilthead sea bream (*Sparus auratus*) during exposure to low salinity (Mancera *et al.*, 1994). Other than plasma osmolality, Na^+ level, plasma cortisol level was elevated when freshwater adult (Dang *et al.*, 2000) or larva (Ayson *et al.*, 1995) tilapia *Oreochromis mossambicus* was treated with cortisol. Such treatment also increased the opercular membrane chloride size and cell density and Na^+-K^+-ATPase density in a dose dependent manner. The apparent association between the elevation of plasma cortisol concentration in freshwater fish and chloride cell proliferation, suggests that cortisol also appears to contribute to the ion uptake mechanism, ionic/osmotic regulation of

freshwater fish in addition to induce ion secretion. It has a dual osmoregulatory function in teleosts

Cortisol treatment in both early and late juvenile coho salmon *Oncorhynchus kisutch* during smoltification stimulated lipid mobilization in parr. Cortisol stimulated lipase activity leading to significant reductions in total lipid concentration and triacylglycerol content of the liver and dark muscles. Mesenteric total lipid concentration, total tissue mass or triacylglycerol content were not affected but mesenteric fat lipase activity was elevated after cortisol administration. Cortisol treatment failed to elicit alterations in the pattern of tissue lipid mobilization of smolts. After cortisol implantation, lipolytic enzyme (triacylglycerol lipase) activity was elevated to decrease liver total lipids content, total lipid concentration, or in total tissue mass (mesenteric fat) and generally resulted in reduced tissue triacylglycerol content. Smolts which characteristically had elevated liver lipase activity, when hypophysectomized, exhibited low levels of liver lipase activity. Cortisol replacement restored enzyme activity to approximately the same levels as those observed in sham-operated controls. These results indicate that cortisol stimulates lipid mobilization in developing salmon by enhancement of lipolysis and suggest that cortisol contributes to smoltification-associated lipid depletion (Sheridan, 1986).

Cortisol has been shown to influence immune functions in a number of fish species. It involves in regulating recovery metabolism after metabolic, acid-base and endocrine disturbances (Milligan, 1996; Narnaware and Woo, 1999). Moreover, after a long-term treatment with cortisol, inhibition on the larval growth occurred.

2.5.4.5. Combined effects of GH, IGF-I, PRL and cortisol

An additive effect of IGF-I and cortisol was found on gill Na^+-K^+-ATPase activity and mRNA levels. A significant effect on the number of Na^+-K^+-ATPase immunoreactive cells (chloride cells) in the gill in the Atlantic salmon (Seidelin *et al.* 1999) was also observed. IGF-I can increase salinity tolerance in short-term treatments in 12 ppt but it is less effective than GH in increasing salinity tolerance and gill Na^+-K^+-ATPase activity in long-term treatments and in interacting with cortisol (McCormick, 1996).

Injection of PRL in combination with cortisol resulted in a marked additive increase in plasma osmolality in hypophysectomized fish relative to that of the sham-operated fish. Cortisol may also promote ion uptake and interacts positively with PRL during acclimation to freshwater (McCorkmick, 2001). PRL did not stimulate cortisol secretion either in hypophysectomized fish or in intact fish. Both PRL and cortisol seem to be involved importantly in ion uptake from the environment not only in fresh water but also in brackish water. PRL also antagonizes the SW-adaptive effect of cortisol (Seidelin & Madsen 1997).

Growth hormone acts in synergy with cortisol to increase gill Na^+-K^+-ATPase activity and salinity tolerance in several salmonid species, *Fundulus heteroclitus* (Mancera and McCormick, 1999), in both hypophysectomized and intact fish in brown trout (Madsen, 1990a). These may be due to the fact that both GH and cortisol are involved in seawater adaptation in stimulating ion extrusion. Growth hormone also acts in several ways: (1) through the upregulation of gill cortisol receptors such

as in coho and Atlantic salmon (Shrimpton *et al.,* 1995; Shrimpton and McCormick, 1998). The number of gill cortisol receptors is strongly correlated with the capacity of cortisol to stimulate gill Na^+-K^+-ATPase *in vitro* and *in vivo* (Shrimpton and McCormick, 1999), indicating that the regulation of cortisol receptors is physiologically relevant. (2) *In vivo* and *in vitro* exposure to GH increases the sensitivity of interrenal tissue to adrenocorticotrohic hormone (ACTH) in coho salmon, causing increased release of cortisol by interrenal tissue (Young, 1988). (3) Corticotropin releasing hormone is a potent stimulator of *in vitro* growth hormone release in European eel (*Anguilla anguilla*) (Rousseau *et al.,* 1999). These all may be the reasons for the synergistic action of the two hormones, GH and cortisol.

The action of cortisol in promoting ion uptake or secretion may therefore depend in part on the relative activity of GH and PRL. Under conditions of high GH and low PRL, cortisol may act primarily to promote salt secretion. Conversely, low GH and high PRL will cause cortiosl to promote ion uptake. This indicated that a combination of these hormones and/or an additional hormone(s) acting in a synchronized fashion may be required for full effectiveness of cortisol function (Richman, *et al.* 1987). Among teleost fishes, individual species and effector organs will differ in the degree to which cortisol interacts with other hormones.

GH and IGF-I alone and in combination, stimulated Na^+-K^+-ATPase α-subunit mRNA content in the gill. Stimultaneous administration of PRL completely inhibited the increase in gill α-mRNA observed in the IGF-I injected groups suggesting an anti-SW effect and a densensitisation of gill filament to IGF-I. Combination of GH and IGF-I did not further affect the α-mRNA level relative to the single hormone-injected

groups (Seidelin and Madsen, 1999). They showed a positive interaction in combination with increasing salinity tolerance, but not gill Na^+-K^+-ATPase activity in *Fundulus heteroclitus* (Mancera and McCormick, 1999). Evidence for the importance of the GH/IGF-I axis in seawater acclimation comes primarily from studies on exogenous hormone treatment, changes in circulating levels, metabolic clearance rate, localization of receptors and production of IGF-I by osmoregulatory tissues. GH and IGF-I stimulate the number and/or size of gill chloride cells in salmonids and tilapia (Sakamoto *et al.*, 1993; Xu *et al.*, 1997). GH and IGF-I increase gill Na^+-K^+-ATPase activity and/or mRNA levels in salmonids, tilapia and mummichog (Madsen *et al.*, 1995; Mancera and McCormick, 1998; Xu *et al.*, 1997; Sakamoto *et al.*, 1997) and immunocytochemical studies indicated that hormone induced increases in Na^+-K^+-ATPase are localized to chloride cells (Seidelin *et al.*, 1999).

The effect of ovine GH *in vivo* and recombinant bovine IGF-I on gill Na^+-K^+-ATPase activity was investigated during the parr-smolt transformation period of coho salmon. The ability of IGF-I *in vitro* to increase gill Na^+-K^+-ATPase activity and the ability of GH to regulate *in vitro* responsiveness of gill tissue to IGF-I further suggests an indirect action of GH on gill tissue and a direct action of IGF-I. The ability may depend on priming by endogenous or exogenous GH. This supports the role of IGF-I as an endocrine mediator for GH action during parr-smolt transformation (Madsen and Bern, 1993). In 1993, Sakamoto and Hirano also found that levels of IGF-I mRNA in gill and kidney increase following GH injection and exposure to seawater, indicating that local production of IGF-I may act in a paracrine fashion to influence transport capacity of gill and renal epithelia. At least some of the osmoregulatory actions of GH are carried out by IGF-I. *In vivo* studies indicate that IGF-I by itself

does not carry out all of the osmoregulatory actions of GH, and that other endocrine factors and/or binding proteins may also be involved. Recombinant bovine (rbIGF-I) had significantly greater salinity tolerance as judged by lower plasma sodium, osmolality, and muscle moisture content when 12 ppt seawater-adapted fish were transfered to 34 ppt. IGF-I administration did not increase salinity tolerance or gill Na^+-K^+-ATPase activity following transfer to 25 ppt SW. Implantation of IGF-I and cortisol in combination significantly increased gill Na^+-K^+-ATPase activity. Although IGF-I can increase salinity tolerance in short-term treatments in 12 ppt, it is less effective than GH in increasing salinity tolerance and gill Na^+-K^+-ATPase activity in long-term treatments and in interaction with cortisol (McCormick 1996).

PRL has a stimulation of hepatic IGF-I mRNA synthesis (Shepherd *et al.*, 1997). Simultaneous administration of PRL completely inhibited the increase in gill Na^+-K^+-ATPase α-subunit mRNA amount in IGF-I injected SW-acclimated trout (Seidelin *et al.* 1999), suggesting a desensitization of the gill tissue to IGF-I and showing the overall anti-SW adaptive effect of PRL. PRL also antagonizes the SW-adaptive effect of GH (Madsen & Bern 1992; Seidelin and Madsen, 1997).

2.6. Salinity effects on Na^+-K^+-ATPase expression

Highly activated chloride cells in branchial and opercular epithelia may be responsible for salt secretion in hyperosmotic environments (Uchida *et al.*, 2000). The synthesis of Na^+-K^+-ATPase and increased number, proliferation and complexity of chloride cells occur prior to the elevation of Na^+-K^+-ATPase activity deputed to salt extrusion (Uchida *et al.*, 1996; Ura *et al.*, 1997). The abundance and activity of gill

Na^+- K^+-ATPase was remarkably increased in response to elevated environmental salinity in salmonids (*Oncorhynchus mykiss:* Madsen and Naamansen, 1989), eels (*Anguilla anguilla:* Motais, 1970), tilapia (*Oreochromis mossambicus:* Hwang *et al.*, 1989), killifish (*Fundulus heteroclitus:* Jacob and Taylor, 1983), and in the vast majority of euryhaline and stenohaline species (Kamiya and Utida, 1969; Thomson and Sargent, 1977; Richman *et al.*, 1987; Lubin *et al.*, 1989). The rise in Na^+-K^+-ATPase hydrolytic activity in gills follows the rise in α-mRNA expression at first, and was then followed by a slower rise in α-protein expression leading to higher synthesis of Na^+-K^+ pumps eventually (D'Cotta H., *et al.*, 2000).

Elevated chloride cell exposure areas were typically found at salinity extremes. Elevations in CC numbers occur in response to higher salinity environments (Kultz *et al.*, 1993). The size of immunoreactive chloride cells was twice larger, with deeply invaginated apical crypt and well-developed tubular network in seawater (SW) than in freshwater (FW). Few studies have reported higher Na^+- K^+-ATPase in FW- than in the SW-acclimated condition such as found in *Chelon labrosus* (Gallis *et al.*, 1979), *Fundulus heteroclitus* (Towle *et al.*, 1977), *Cyprinodon salinus* (Stuenkel and Hillyard, 1980), *Gillichthys mirabilis* (Doneen, 1981) and *Maqcuaria novemaculata* (Langdon, 1987). In a few species, similar Na^+- K^+-ATPase activities were found in FW and SW so that Na^+-K^+-ATPase was usually but not always, proportional to the external salinity (*Gillichthys mirabilis:* Yoshikawa *et al.*, 1993; *Morone saxatilis:* Madsen *et al.*, 1994; McCormick, 1995).

Gill Na^+-K^+-ATPase α-subunit mRNA reached maximum together with peak intensity of immunoreactivity for Na^+-K^+-ATPase α-subunit protein in branchial

chloride cells and elevation of Na^+-K^+-ATPase activity near the time of parr-smolt transformation. The parr-smolt transformation is the most active period in salmonids, and represents a developmental stage characterized by preparation for seaward migration (Zaugg and McLain, 1970, 1972; Langhorne and Simpson 1986; McCormick *et al.*, 1989; D'Cotta *et al.*, 1996; Hwang, 1998). It is used also as an indicator of transformation. Non-migratory salmonids had significantly lower gill Na^+-K^+-ATPase activity than that of migrant fish of the same species. The relationship has been found in hatchery-reared Chinook salmon, *Oncorhynchus tshawytscha* (Hart *et al.*, 1981), wild coho salmon, *Oncorhynchus kisutch* (Rodgers *et al.*, 1987; Ewing and Rodgers, 1998), Atlantic salmon, *Salmo salar* L. (McCormick and Bjornsson, 1994), and hatchery-reared steelhead (Zaugg, 1981; Ewing *et al.*, 1984). The increase in Na^+-K^+-ATPase activity during smoltification may be explained by (1) the pre-adaptation hypothesis (Boeuf, 1993) and (2) the ion imbalance hypothesis (Thorpe, 1989). In the pre-adaptation hypothesis, seaward migration of juveniles is thought to stimulate the increase in the ion-pumping machinery of the gills necessary for SW adaptation. In this hypothesis, seaward migration in long river systems should begin before the increase in Na^+-K^+-ATPase activity. In the ion imbalance hypothesis, seaward migration of the juveniles results from changes in kidney function that causes the loss of internal sodium ions. Gill Na^+-K^+-ATPase activity is therefore increased to improve the influx of sodium ions and restore internal sodium concentrations. At the same time, the fish loses the ability to maintain its position in the river. In this hypothesis, seaward migration and increases in gill Na^+-K^+-ATPase activity should begin simultaneously, as the juveniles begin to float seaward with the river current (Lysfjord and Staurnes, 1998).

Gill Na^+-K^+-ATPase activity of the European sea bass *Dicentrarchus labrax* was unchanged in 15 ppt (ISO) but doubled in FW- and in 50 and 60 ppt seawater-groups (high salinity seawater). In both groups, this was preceded by a 2- to 5- fold elevation of the gill α-subunit Na^+-K^+-ATPase mRNA level. Thus, increased expression of α-subunit mRNA is part of the molecular mechanism of both FW and SW acclimation in sea bass. Gill Na^+-K^+-ATPase Na^+-, K^+-, and ouabain-affinity were similar in fish acclimated to FW, 15 ppt, and high salinity SW, suggesting that identical isoforms of the catalytic subunit of the enzyme are expressed regardless of ambient salinity (Jensen, *et al.*, 1998).

There were no visual sign of stress in European sea bass transferred to 50 ppt, 25 ppt, 15 ppt, 5 ppt SW and 0 ppt FW. Plasma osmolality was lower in the FW group and higher in the 50 ppt and 60 ppt groups relative to the 15 ppt control group. Gill Na^+-K^+-ATPase activity was elevated in the FW group and in the 50 ppt and 60 ppt SW groups when compared with the control group (Jensen, *et al.*, 1998). Plasma glucose levels were significantly higher in FW and ISO compared to SW values. It may be related to the elevation of oxygen consumption rates in SW after transfer to SW when compared to those of FW and ISO, suggesting that more energy is required for SW acclimation (Morgan *et al.*, 1997). Oxygen consumption has commonly been used as an indirect indicator of metabolism in fish and measurements of oxygen consumption rates in different salinities have been employed in an attempt to determine the energetic cost of osmoregulation. Gill Na^+-K^+-ATPase activity and oxygen consumption rates of swimming fish are at a minimum in 15 ppt SW in several studies (*Oreochromis mossambicus*: Kultz *et al.*, 1992; *Dicentrarchus labrax:* Jensen, *et al.*, 1998). It is because the numerical osmotic gradient between blood and

water is minimum at ISO salinity. The activity increases after transfer to hypo- or hypersaline conditions. The physiological changes associated with SW acclimation in fish represents a significant short-term energetic cost, the adaptation seeks to minimize the energetic cost of osmoregulation (as much as 20% of total body metabolism in SW). If fish can maintain ion-osmotic homeostatsis with lowest possible Na^+-K^+-ATPase activity, more energy is available for growth (Febry and Lutz, 1987; Woo *et al.*, 1997; Jensen, *et al.*, 1998). However, when tilapia *Oreochromis mossambicus* were reared from yolksac fry for 20 months in FW or SW, the oxygen consumption rate in SW tilapia was half of that measured in FW fish (Ron *et al.*, 1995). As a conclusion, metabolic cost of acclimating to SW may be high in the short-term, but that in a fully-adapted tilapia the energy requirements of a marine existence may be lower than in FW.

Numerous studies have addressed the influence of salinity on the growth rate of fish (Teskerezic et al., 1989; Kuwaye et al., 1993; Woo *et al.*, 1997). The fish may grow better under isosmotic conditions compared with the fish acclimated to a hyposmotic or hyperosmotic environment, the final body weights of fish in 33‰ and 6‰ were not significantly different.

The transition from FW to SW is associated with a temporary elevation in plasma osmolality and Na^+ and Cl^- ion concentrations (Assem and Hanke, 1979; Hwang *et al.*, 1989), which was accompanied by a transient rise in plasma cortisol and GH levels in SW (Assem and Hanke, 1981; Hwang, 1987; Yada *et al.*, 1994; Morgan *et al.*, 1997); and both plasma PRL levels (PRL_{177} and PRL_{188}) decrease in ISO and SW compared to FW (Morgan *et al.*, 1997). The ability to osmoregulate in

SW appears to be closely associated with the secretion of GH and cortisol suggesting that salinity induced multihormonal effect on osmoregulation.

Chapter 3

In vitro effect of hormones on branchial Na^+-K^+-ATPase expression in marine teleost *Sparus sarba*

3.1 Abstract

Na^+-K^+-ATPase is the primary driving force of trans- and paracellular movements of monovalent ions in both freshwater and seawater adapted fish gills. It is known that the enzyme protein consists of a catalytic α-subunit and a β-subunit but the hormonal regulation of subunit expression remains unclear. This study attempted to relate the effects of key osmoregulatory hormones on branchial Na^+-K^+-ATPase subunit expression in the silver sea bream *Sparus sarba*. The effects of recombinant bream growth hormone (rbGH), recombinant bream insulin-like growth factor-I (rbIGF-I), ovine prolactin (oPRL) and cortisol (Co) were studied *in vitro* by incubating branchial filaments with different doses of hormone.

For mRNA subunit analysis, total RNA from branchial tissue was analyzed by using silver seabream Na^+-K^+-ATPase α- and β-subunit cDNA clones as radiolabelled probes. rbGH treatment (1ng/ml) significantly increased both α-mRNA subunit and β-mRNA subunit abundance when compared with the saline-treated controls. rbIGF-I treatment markedly increased α-mRNA subunit at all doses of rbIGF-I added (1, 10, 100 or 1000ng/ml) and reached maximum at 1ng/ml rbIGF-I. rbIGF-I treatment significantly decreased β-mRNA subunit at higher doses (10, 100 or 1000ng/ml) in a dose dependent manner. Both oPRL and Co treated groups caused no significant

changes in both branchial Na^+-K^+-ATPase α- and β-mRNA subunits at all doses under the conditions of this experiment.

For protein subunit analysis, only Na^+-K^+-ATPase α-protein subunit was investigated. Both rbGH and rbIGF-I revealed dose-response effect on the expression, with a lower elevation of the Na^+-K^+-ATPase α-protein subunit expression as dose of hormone increased. rbGH significantly elevated α-protein subunit level at 1 and 10 (2-2.3 fold) when compared with saline-treated control. 1ng/ml rbIGF-I displayed a marked (6.6 fold) increase in the subunit abundance and an elevation of 3 fold increase in the level when treated with 10ng/ml rbIGF-I. Both oPRL and Co caused no significant change in the protein subunit under the conditions of this experiment.

Both rbGH and rbIGF-I treated groups showed a bell shape or parabolic dose-dependent effect on Na^+-K^+-ATPase functional activity. rbGH treated increased when 1ng/ml hormone was added and reached a maximum value at 10ng/ml and decreased as the concentration of hormone increased to 100 and 1000ng/ml. However, significant change in Na^+-K^+-ATPase activity was only observed at 10ng/ml. rbIGF-I increased Na^+-K^+-ATPase activity at lower doses (1 and 10ng/ml rbIGF-I) higher doses caused the activity to decrease to the level of saline-treated controls. oPRL and Co had no significant effect on Na^+-K^+-ATPase activity under the conditions of this experiment.

The data suggest that *Sparus sarba* branchial Na^+-K^+-ATPase expression is sensitive to *in vitro* regulation by rbGH and rbIGF-I and both hormones induce transciption, translation and posttranslation of Na^+-K^+-ATPase.

3.2 Introduction

Active transport and secretion of ions, especially sodium, potassium, bicarbonate and chloride, occur across the epithelial membranes of the osmoregulatory tissues such as gills. Such membranes are osmotically permeable to water, in which both water and ions can pass across depending on the membrane and the physiological conditions. Hormones can act directly on the cell involved and influence the ion transport activity of osmoregulatory tissues through their physiological actions. Alterations in secretion and absorption of ions across the gills of fishes may be modulated and even controlled by hormones primarily (Sakamoto *et al.*, 1997; Fernandes *et al.*, 1998; Seidelin and Madsen, 1999; Kelly *et al.*, 1999c; Eckert *et al.*, 2001).

Many studies have been conducted on the hormonal control of osmoregulation in teleosts. It has been generally accepted that prolactin (PRL) is the dominant factor in regulating hydromineral balance in freshwater and cortisol is the dominant factor in seawater (Utida *et al.*, 1972; Hirano, 1986; Brown and Brown, 1987; Bern and Madsen, 1992; McCormick, 1995, 2001). Growth hormone (GH) and its mediator, insulin-like growth factor-I (IGF-I) have also been confirmed to enhance the adaptation of several salmonids and non-salmonids to seawater (Sakamoto and Hirano, 1993; McCormick, 1995; Shepherd *et al.*, 1997; Mancera and McCormick, 1999). Several studies have shown that, in FW-acclimated fish, growth hormone (GH), insulin-like growth factor-I (IGF-I) and cortisol increase salinity tolerance and increase primary filament chloride cell size and number (Madsen 1990a,b; Laurent *et al.* 1994, Seidelin & Madsen 1997). The induction or impairment of the expression of

enzyme such as Na^+-K^+-ATPase that carries out key physiological roles could cause alterations of the physiology of the whole organism. However, It is still not known how hormones affect biosynthesis and regulation of Na^+-K^+-ATPase mRNA, protein subunit and functional activity. The present study was performed to obtain a better understanding of the molecular mechanisms underlying the hormonal effect on branchial Na^+-K^+-ATPase *in vitro*.

In vitro study could be useful in the understanding of the effects on aquatic organisms *in vivo*. For example, Na^+-K^+-ATPase enzymatic activities were inhibited by the heavy metal cadmium when the activity was measured in eel intestinal and branchial homogenates *in vitro*, with the branchial activity being more sensitive to the action of the heavy metal than the intestinal one (Lionetto *et al.*, 2000). In the same way, direct hormonal effect on the expression can also be investigated *in vitro*. *In vitro* studies mimic many of the passive transport and electrophysiological characteristics. Addition of hormone to gill tissue *in vitro* is a useful methodology to assess whether a single factor is sufficient to stimulate changes in tissue physiology.

The present study is to investigate the *in vitro* effect of four hormones including recombinant bream growth hormone (rbGH), recombinant bream insulin-like growth factor-I (rbIGF-I), ovine prolactin (oPRL) and cortisol (Co) on silver seabream *Sparus sarba* branchial Na^+-K^+-ATPase expressions.

3.3 Materials and methods

3.3.1. Overall experimental design

In this experiment, fresh gill filaments were cut from silver sea bream and incubated with different hormones in Dubelco's Minimal Essential Medium (DMEM) medium for 2hr at room temperature. Half of the treated gill filaments was used for Na^+-K^+-ATPase mRNA subunits analysis by using dot blotting, another half was used for Na^+-K^+-ATPase α-protein subunit and Na^+-K^+-ATPase activity analysis.

3.3.2. Fish preparation

Silver sea bream (*Sparus sarba*), weighing between 95-156g, were obtained from local sea cages (33ppt) and allowed to acclimate to culture conditions in recirculating seawater (SW, 33ppt) filtration systems for 3 weeks in The Marine Science Laboratory, The Chinese University of Hong Kong. Temperature was held between 18 and 22°C and the fish were exposed to a natural photoperiod (11h L :13h D) and the salinity of the water during this period was 33-34ppt. Fish were fed once daily with a pellet diet formulated by Woo & Kelly (1995). Feeding was terminated 24 hr prior to sacrifice.

3.3.3. Tissue sampling

Fish were killed by spinal transection and the heads were prepared for sampling of gill tissue. Gill tissues intended for RNA analysis, protein analysis and

enzyme activity measurements, were not perfused and were removed from the fish immediately after decapitation. The three anterior pairs of gill arches were used and incubated in fresh DMEM (GibcoBRL) medium with a particular concentration of hormone (0, 1, 10, 100 or 1000ng hormone/ml of DMEM). The hormone was added directly into the Petri dishes and the preparation was incubated at ambient temperature of 19°C for 2 hr with gentle shaking. Four hormones were used in the experiment including recombinant bream growth hormone (rbGH) (GroPep Pty Ltd, Australia), recombinant bream insulin-like growth factor-I (rbIGF-I) (GroPep Pty Ltd, Australia), ovine prolactin (oPRL) (from sheep pituitary glands; Sigma, USA) and cortisol (Co) (hydrocortisone; Solu-Cortef; Upjohn, Belgium).

Each hormone treatment group was divided into four subgroups (n=8) at four different concentrations (1, 10, 100 and 1000ng/ml). The hormones were dissolved in sterilized 0.85% saline and diluted in fresh DMEM to these four concentrations. A same amount of 0.85% saline was added into fresh DMEM served as the control group (0ng/ml hormone concentration). Each treated gill arch was cut into two pieces, one for RNA analysis and another for protein analysis and enzyme assay, and were immediately frozen in liquid nitrogen and stored in deep freezer at -70°C for subsequent analysis.

3.3.4. RNA extraction and dot blot analysis

The gill filaments were trimmed from the gill arches and were homogenized for 10sec in 1ml of lysis buffer (5M guanidine thiocyanate, 9mM EDTA, 45mM Tris/HCl pH 8.0) by using an Ultra-Turrax T25 rotor stator homogenizer (IKA

Labortechnik, Germany). Total RNA was extracted from the lysate using a Qiagen RNeasy mini kit (Qiagen, GmbH, Germany), aliquoted and stored at –70°C.

RNA dot blots were prepared using a Bio-Dot microfiltration manifold (BioRad, USA). Branchial RNA samples (4μg) from individual fish, were prepared according to instructions supplied with Hybond N+ membrane. Each sample was prepared in triplicate onto three separate membranes and each membrane was probed with *Sparus sarba* branchial Na^+-K^+-ATPase α, β subunit cDNA clones respectively. The mRNA on dot blots were hybridized with *Sparus sarba* branchial Na^+-K^+-ATPase α and β subunit cDNA clones that were isolated and characterized by Dr. Eddie E. Deane (Deane, *et al.*, 1999), the sequences of which were shown in Figs. 3.1 and 3.2. Radiolabeled subunit cDNA probe was prepared using a Rediprime random labeling kit (Amersham, UK). The dot blots were hybridized in Rapid-Hyb buffer (Amersham, UK) at 55°C for 16hr. After hybridization, the membrane was washed twice with 2X SSC, 0.1%SDS solution, for 15min at 55°C and then once in a 0.1X SSC, 0.1% SDS solution for 15min at 55°C. The dot blots were then exposed to a storage phosphor screen (Molecular Dyamics, USA) for 3hr at room temperature. The plates were scanned using the Storm Phosphor Imaging system with Image Quant software (Molecular Dynamics, USA) allowing for quantification of mRNA transcript. Subunit mRNA abundance was normalized to its corresponding 18S rRNA abundance for each sample.

3.3.5. Protein extraction

The gill filaments were trimmed from the gill arches and were homogenized by Ultra-turrax homogenizer in ice cold SEI (150mM sucrose, 10mM EDTA, 50mM imidazole, pH 7.5)/ SEID (0.5g sodium deoxycholate in 100ml of SEI) buffer. The ratio of SEI to SEID was 4:1.

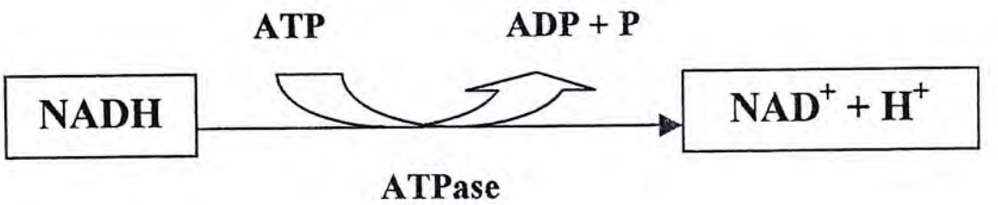
3.3.6. Protein quantification

The total soluble protein concentrations of the supernatants were quantified by using the protein-dye binding method of Bradford (1976). First of all, 20µl of the supernatants was hydrolyzed in 80µl 0.1M NaOH at room temperature for 3 hours. 20µl of the mixture was mixed with 1ml filtered Bradford solution (1L solution contains: 0.4g Brilliant Blue G-250 dissolved in 50ml ethanol (96%), 100ml phosphoric acid H_3PO_4 (85%) in distilled water). The absorbance of the samples was read by spectrophotometer (Milton Roy Spectronic 3000 Array) at 595nm. The total soluble protein concentrations of the samples were quantified by calibrating with several dilutions of protein standard solutions of bovine serum albumin (BSA; Sigma) with concentration between 0 and 1 mg/ml.

3.3.7. Na^+-K^+-ATPase activity

Na^+-K^+-ATPase activity was measured according to the principle as laid out in McCormick (1993). The protein content of supernatants was determined using bovine serum albumin (Sigma) as a standard. Enzyme activity was measured at 24°C and

expressed as protein specific activity. The reaction follows the principle that Na^+-K^+-ATPase oxidizes NADH to NAD^+ by using ATP as the energy source. Measurement of the decrease in NADH level indicates the total enzyme activity. Specific activity of Na^+-K^+-ATPase, can be obtained by using its inhibitor ouabain. Thus, specific Na^+-K^+-ATPase activity can be represented as the difference between the total enzyme activity (without ouabain added) and the enzyme activity after addition of ouabain.



3.3.8. Protein gel electrophoresis and immunoblotting (Western blotting)

After measuring the Na^+-K^+-ATPase activity, the enzyme extracts were used for protein subunit analysis by Western blotting. Laemmli one-dimensional SDS-PAGE was firstly applied to resolve proteins of different molecular sizes (Laemmli, 1971). A 4% stacking and a 12% separating SDS polyacrylamide gel were used. 10μg of total soluble protein of samples were mixed with loading buffer (1.2% Tris base, 10% SDS, 10%-mercaptoethanol, 20% glycerol and 0.1% bromophenol blue, pH6.8) and were denatured at 97°C for 10min before loaded into wells and electrophoresed by a Bio-Rad mini gel kit for 1hr at 140V.

The resolved proteins were transferred from the separating gel to a nitrocellulose membrane (GibcoBRL), by an electrotransfer cell (Bio-Rad) for 90mins at 150V. The membrane was then air-dried for a few minutes and blocked in 0.01M

phosphate-buffered saline (PBS, pH7.4) containing 0.05% Tween-20 (PBS-T) with 3% skimmed milk powder overnight at 4°C. The membrane was then incubated with two layers of specific antibodies. The primary antibody was mouse anti-chicken α5 Na^+-K^+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa) and the secondary antibody was sheep anti-mouse IgG, horseradish peroxidase linked whole antibody GPR (Amersham Life Science). Primary antibody was diluted 1:4,000 in PBS-T and secondary antibody was diluted 1:10,000 in PBS-T. In between the incubations, the membrane was washed for 15min three times in PBS-T. Finally, the Na^+-K^+-ATPase α-protein subunit band of the samples was visualized using the ECL development system (Amersham International plc), onto the membrane. The signals can be shown by exposed membrane in lumi-imager F1 (Roche) for 1min.

3.3.9. Statistical analysis

All data are expressed as mean values ± S.E.M. The groups were subjected to a one-way analysis of variance (ANOVA) to test for significance followed by a Student–Neuman–Keuls multiple comparison test (Jandel Scientific) to delineate significance between groups.

```
V   T   G   D   H   P   I   T   A   K   A   I   P
GTT ACT GGG GAC CAT CCG ATA ACT GCT AAG GCT ATC CCT -39
K   G   V   G   I   F   S   E   G   N   Q   T   V
AAG GGG GTG GGT ATC TTC TCC GAA GGC AAC CAG ACT GTT -78
E   D   I   A   A   R   L   N   V   P   I   S   E
GAA GAT ATT GCT GCC CGC TTG AAT GTA CCC ATC TCA GAG -117
V   R   P   R   D   A   K   A   C   V   V   H   G
GTG AGG CCC AGG GAC GCC AAG GCC TGC GTT GTC CAC GGT -156
G   L   L   K   D   L   S   A   E   I   F   D   Y
GGC CTC CTG AAA GAC CTG TCA GCC GAA ATT TTT GAT TAT -195
I   F   V   H   H   T   E   I   V   F   A   R   T
ATT TTT GTC CAC CAC ACT GAA ATT GTG TTT GCC AGA ACA -234
S   R   Q   Q   K   L   I   I   V   E   S   C   Q
TCT CCT CAG CAG AAG CTG ATC ATT GTG GAA AGT TGC CAG -273
R   Q   G   A   I   V   A   G   T   G   D   G   G
CGT CAA GGT GCT ATT GTA GCT GGA ACA GGG GAT GGT GGG -312
N   E   S   P   A   L   K   K   A   D   I   W   A
AAT GAA TCT CCT GCC CTG AAG AAG GCT GAC ATA TGG GCC -351
A   I   G   F   S   G   L   E   F   S   K   Q   A
GCC ATT GGT TTT TCT GGA TTG GAA TTT TCC AAA CAA GCC -390
A   D   M   I   L   V   D   D   N   F   A   S   V
GCC GAT ATG ATC CTG GTG GAT GAC AAC TTT GCC TCC GTG -429
V   S   G   V   E   E   G   R   L   I   L   D   N
GTT TCA GGA GTT GAA GAA GGC CGC CTG ATC TTG GAC AAC -468
Q   K   K   S   M   A   Y   I   E   T   S   N   I
CAG AAG AAA TCC ATG GCC TAC ATT GAG ACC AGC AAT ATC -507
P   E   I   S   P   F   L   L   F   I   M   A   N
CCG GAG ATC TCA CCC TTC CTC CTC TTC ATC ATG GCC AAC -546
I   H   R   P   L   G   T   V   T   I   L   C   M
ATC CAT AGG CCC CTG GGA ACC GTC ACC ATC CTC TGT ATG -585
D   L   G   T   D   M   V   P   A   I   S   L   A
GAC CTG GGA ACT GAC ATG GTG CCC GCC ATC TCC CTG GCC -624
Y   E
TAT GAG                                             -630
```

Figure 3.1. Nucleotide and deduced amino acid sequence of *Sparus sarba* branchial, 630bp Na^+-K^+-ATPase α-subunit cDNA clone. Nucleotides are numbered on the left-hand side and the PCR primer regions are underlined (Deane *et al.*, 1999).

```
F   Y   G   C   L   A   G   I   F   I   G   T   I
TTC TAT GGG TGC CTG GCG GGG ATC TTC ATC GGG ACC ATC -39
Q   A   M   L   P   T   L   S   A   Y   K   P   T
CAA GCC ATG CTG CCC ACC CTG AGC GCC TAC AAG CCC ACC -78
W   Q   D   R   V   A   P   P   G   L   T   H   T
TGG CAG GAC AGA GTT GCA CCC CCT GGC CTT ACA CAC ACC -117
P   K   S   D   K   A   E   V   A   F   N   V   N
CCA AAA TCA GAC AAA GCT GAA GTG GCC TTC AAC GTG AAC -156
D   V   E   T   Y   V   P   Y   T   K   A   L   K
GAC GTG GAG ACC TAC GTG CCT TAC ACC AAG GCC TTG AAG -195
E   F   L   P   K   Y   D   D   E   V   Q   R   D
GAG TTC CTG CCC AAG TAT GAC GAT GAA GTC CAG AGG GAC -234
Q   M   K   F   E   D   C   G   D   E   P   G   E
CAG ATG AAG TTC GAG GAT TGC GGA GAT GAG CCT GGA GAG -273
Y   K   N   R   G   D   L   E   S   D   E   G   V
TAC AAG AAC CGC GGC GAC TTG GAG AGC GAC GAG GGT GTC -312
R   K   A   C   R   F   P   R   S   P   L   G   T
AGG AAG GCC TGC CGG TTT CCC AGG TCC CCG CTG GGA ACC -351
L   L   W   H   Q   E   P   Q   F   G   F   K   D
CTG CTC TGG CAT CAG GAA CCG CAG TTT GGC TTC AAG GAC -390
G   K   P   C   F   I   V   K   L   N   R   I   V
GGC AAG CCC TGC TTC ATT GTG AAG CTC AAC AGG ATT GTC -429
N   F   R   P   R   P   P   T   S   N   F   S   I
AAC TTC CGT CCA AGG CCT CCT ACC TCT AAC GAA AGC ATT -468
P   E   E   A   Q   P   K   V   Q   P   N   V   I
CCT GAA GAG GCT GAA CCC AAG GTG CAA CCC AAC GTC ATC -507
P   I   H   C   T   N   K   K   E   E   D   G   W
CCT ATC CAC TGC ACC AAC AAG AAA GAG GAG GAC GGT TGG -546
K   N   R   G   D   Q   V   Y   G   I   G   G   G
AAG AAT CGG GGA GAT CAA GTA TAC GGT ATC GGC GGC GGC -585
F   P   L   Q   Y   Y
TTC CCC CTC CAG TAC TAC CC                        - 605
```

Figure 3.2. Nucleotide and deduced amino acid sequence of *Sparus sarba* branchial, 605bp Na^+-K^+-ATPase β-subunit cDNA clone. Nucleotides are numbered on the left-hand side and the PCR primer regions are underlined (Deane *et al.*, 1999).

3.4. Results

3.4.1. Dot blot analysis of Na^+-K^+-ATPase mRNA subunits

In order to assess the effects of hormone treatment on subunit mRNA abundance, RNA dot blot analysis was used.

The effect of rbGH on branchial Na^+-K^+-ATPase α- and β-mRNA subunits expression were investigated. Treatment of gills with 1ng/ml rbGH significantly increased α-mRNA subunit abundance by 1.7 fold in comparison to saline-treated control group but it caused no significant effect on the subunit expression at higher doses (10, 100, 1000ng/ml rbGH) (Fig. 3.3). At the same time, 1ng/ml rbGH treatment significantly increased 1.9 fold in β-subunit mRNA abundance when compared with saline-treated control. Higher dose of rbGH seems to reduce β-subunit mRNA level in a dose dependent manner when compared with saline-treated group, however, these apparent declines are not significant (Fig. 3.4).

Figures 3.5 and 3.6 show the effect of rbIGF-I on branchial Na^+-K^+-ATPase α- and β-mRNA subunit expression respectively. rbIGF-I elevated branchial Na^+-K^+-ATPase α-mRNA subunit levels at all doses. Dot blot analysis showed a marked elevation in α-subunit level when 1ng/ml rbIGF-I was added in which there was more than ~2.5 fold increase in relative abundance. Gills treated with higher doses showed only a 1.5-1.7 fold elevation in α-mRNA subunit relative abundance. On the other hand, our results revealed a negative effect of rbIGF-I on branchial Na^+-K^+-ATPase β-mRNA subunit. Higher doses (10, 100, 1000ng/ml) of rbIGF-I significantly reduced

β-mRNA subunit dose dependently when compared with saline-treated control (0.5-0.7 fold of control group).

On investigating the effect of oPRL on branchial Na^+-K^+-ATPase α- and β-mRNA subunits expression, the results revealed that oPRL had no significant effect on both mRNA subunits (Figs. 3.7, 3.8). Cortisol also did not cause any change in both of the branchial Na^+-K^+-ATPase α- and β-mRNA subunits expression in *Sparus sarba* (Figs. 3.9, 3.10). There was no significant difference in relative abundance of α- and β-mRNA between hormone-treated and saline-treated fish in both oPRL and cortisol groups.

The stoichiometric ratios of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunit abundance for saline-treated controls and different doses of hormone treatment were measured and are presented in Table 3.1. Treatment with higher doses of rbGH (100 and 1000ng/ml) significantly elevated the ratio of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunit abundance which indicating α-mRNA subunit is more sensitive to rbGH than β-mRNA subunit. All doses of rbIGF-I treatment increased the ratio markly and reached ~3 fold increase at high doses (100 or 1000ng/ml) compared with saline-control. Both oPRL and cortisol did not show any significant different in the ratio of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunit abundance compared with the saline-treated group.

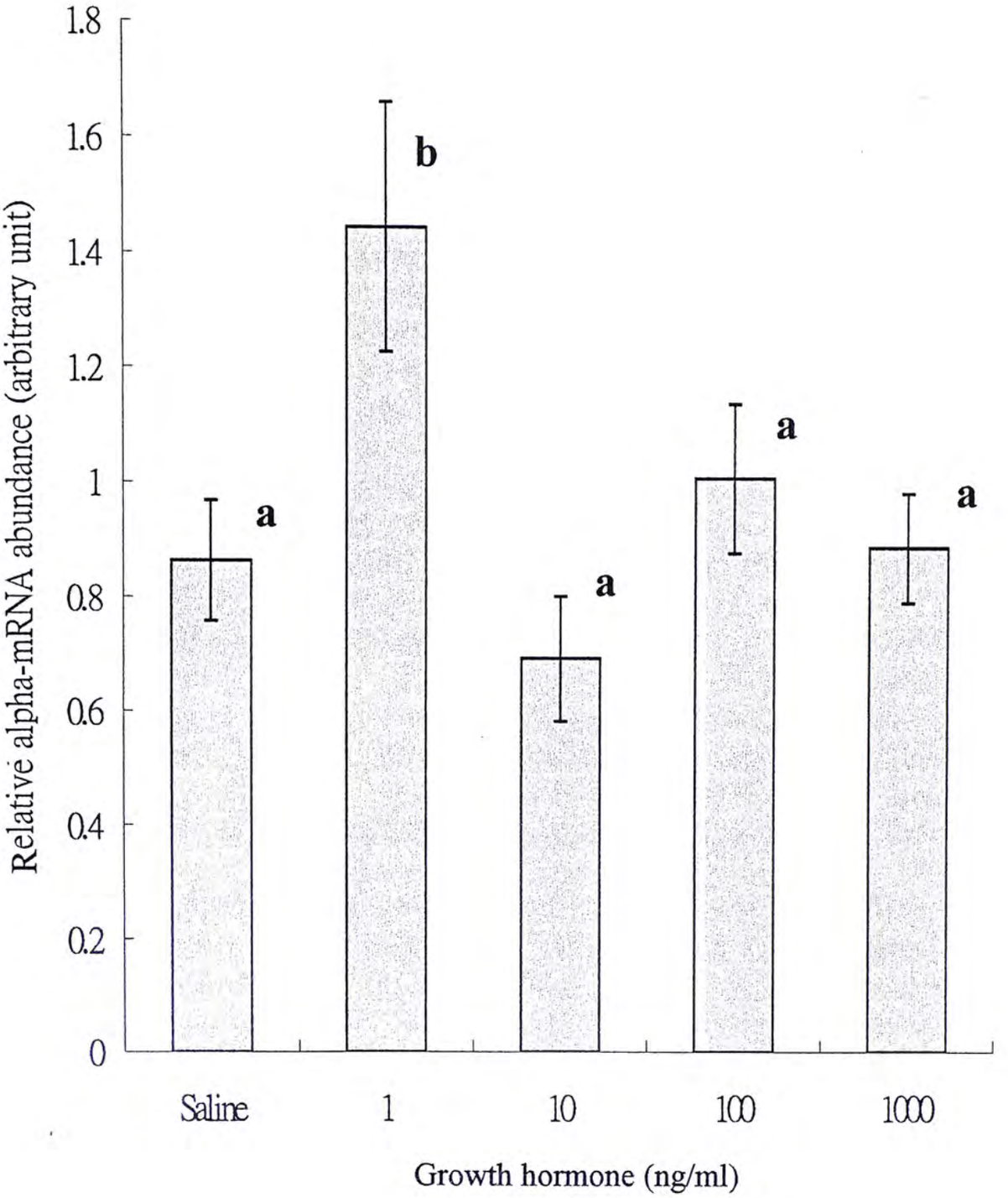


Figure 3.3. *Sparus sarba*: Branchial Na^+-K^+-ATPase α-mRNA after treatment with saline and different doses of recombinant bream growth hormone (rbGH) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-mRNA level (n=6-8). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison. All data were normalized against 18S rRNA.

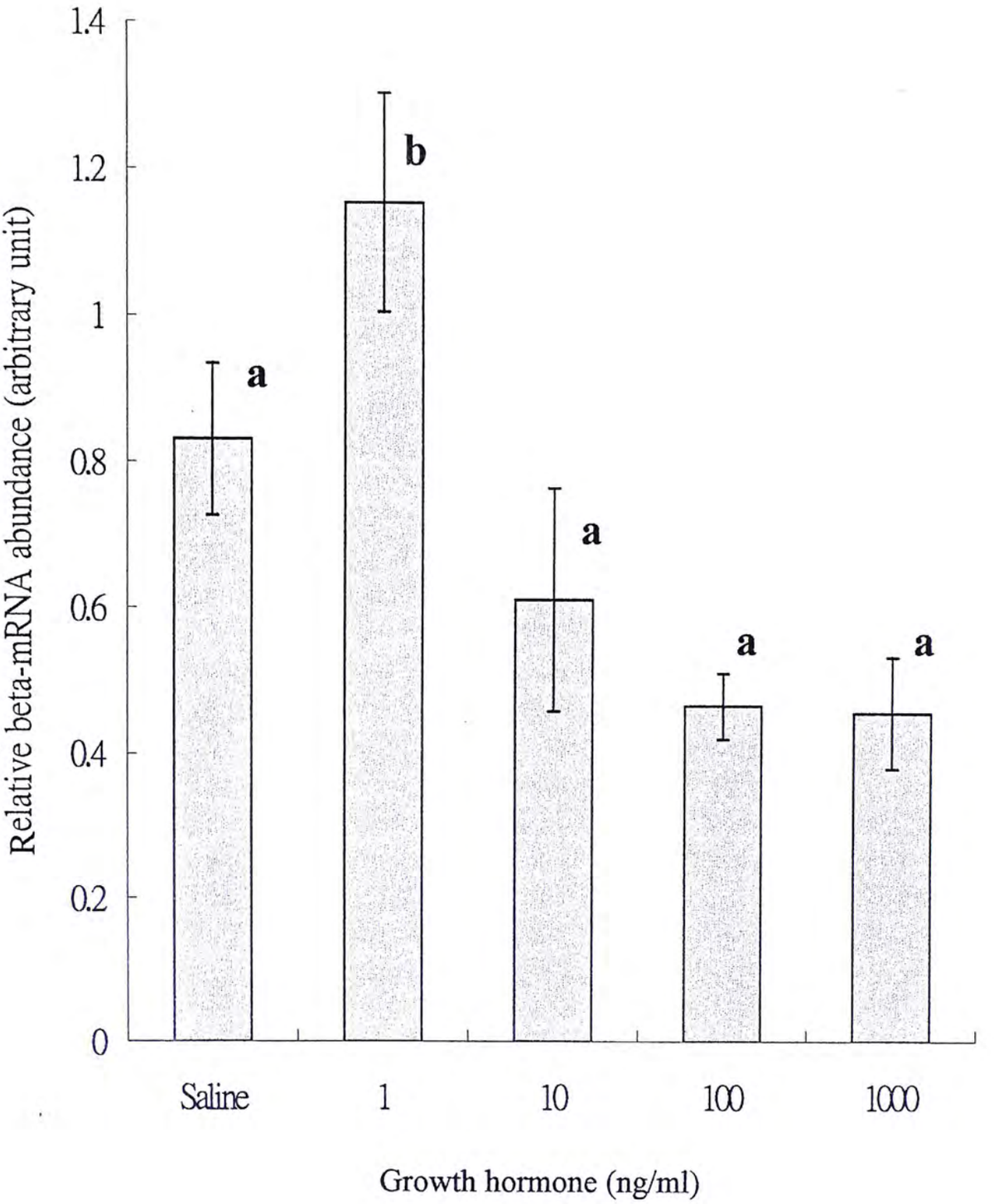


Figure 3.4. *Sparus sarba*: Branchial Na^+-K^+-ATPase β-mRNA after treatment with saline and different doses of recombinant bream growth hormone (rbGH) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase β-mRNA level (n=7-8). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison. All data were normalized against 18S rRNA.

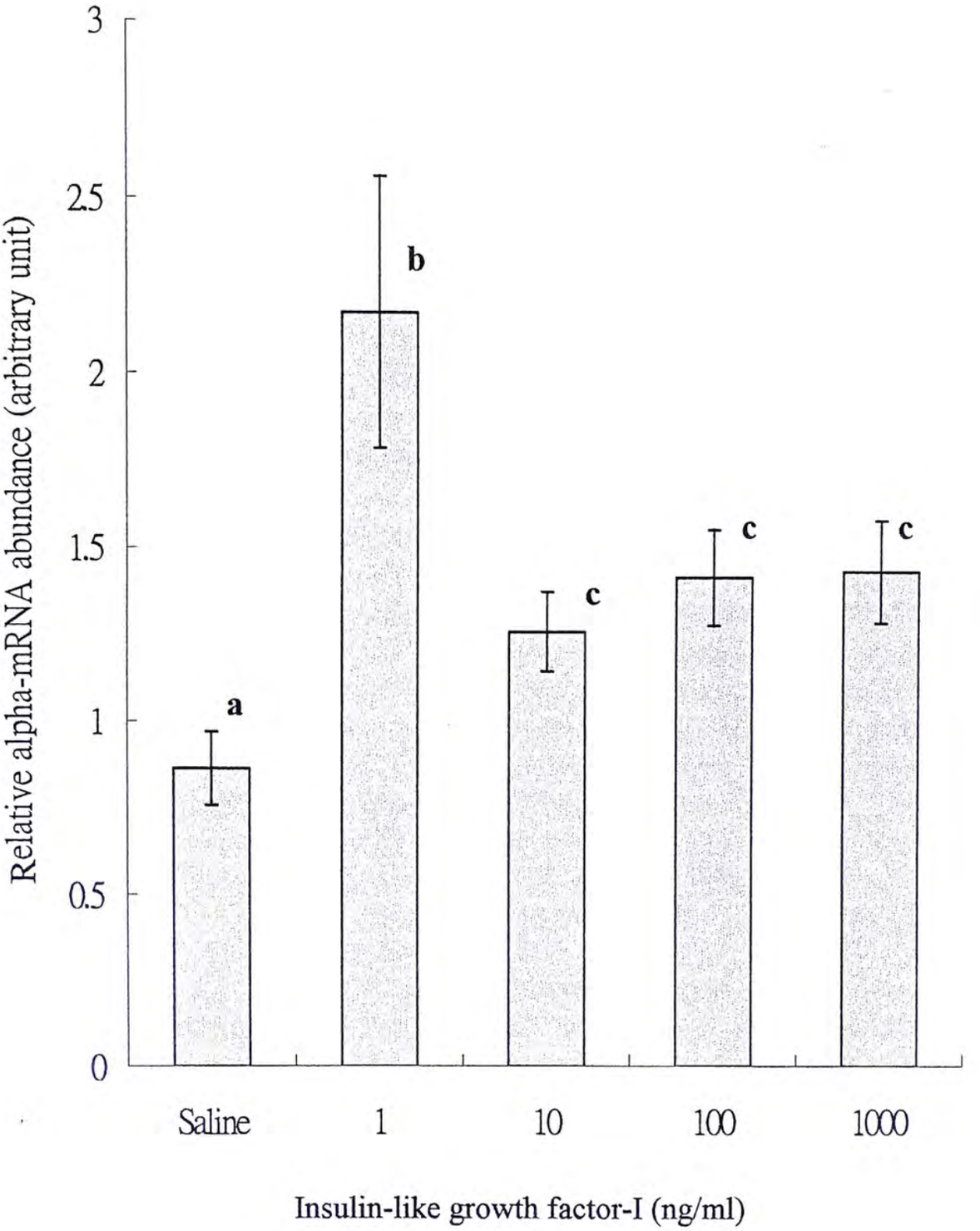


Figure 3.5. *Sparus sarba*: Branchial Na^+-K^+-ATPase α-mRNA after treatment with saline and different doses of recombinant bream insulin-like growth factor-I (rbIGF-I) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-mRNA level (n=8). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison. All data were normalized against 18S rRNA.

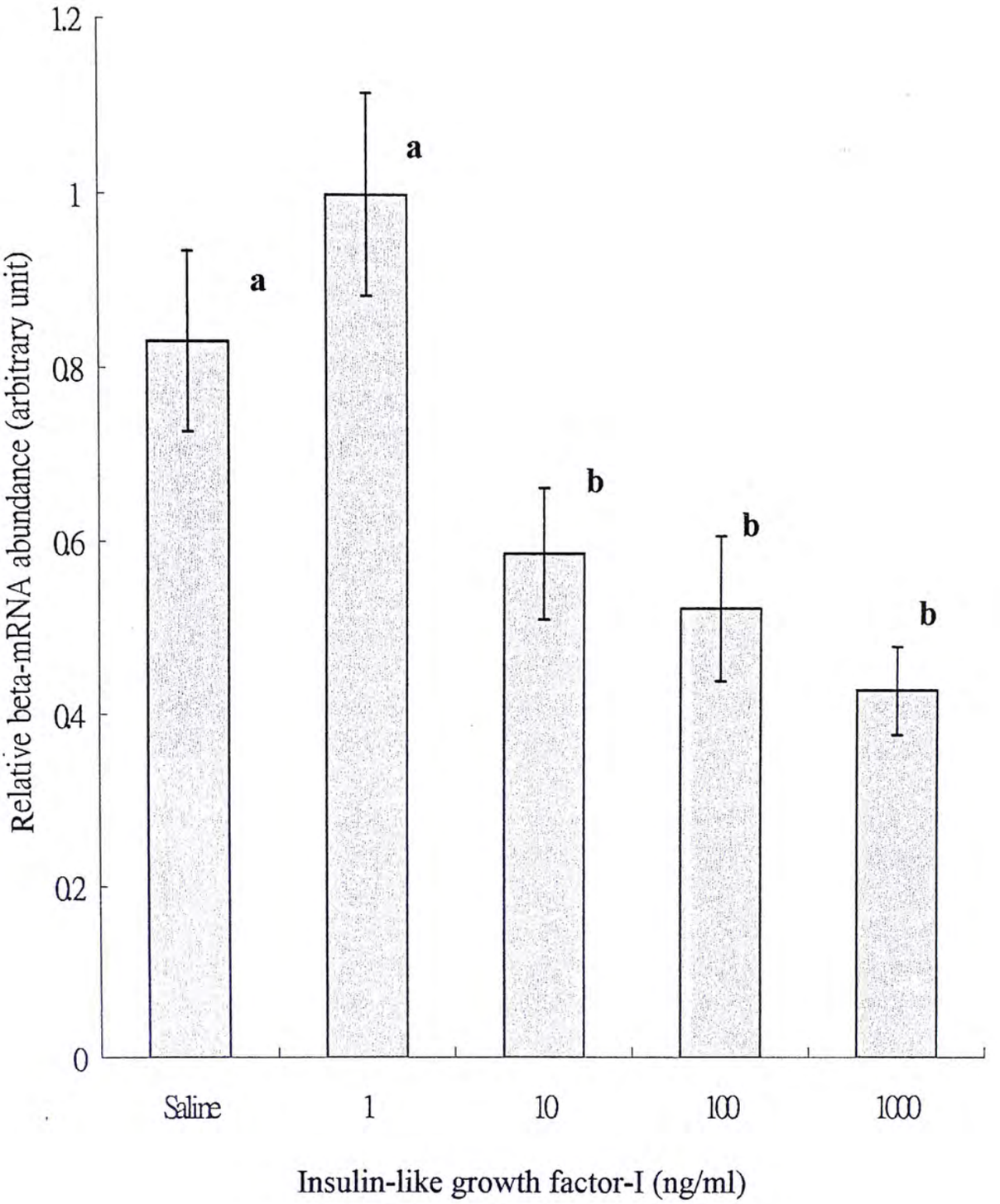


Figure 3.6. *Sparus sarba*: Branchial Na^+-K^+-ATPase β-mRNA after treatment with saline and different doses of recombinant bream insulin-like growth factor-I (rbIGF-I) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase β-mRNA level (n=7-8). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison. All data were normalized against 18S rRNA.

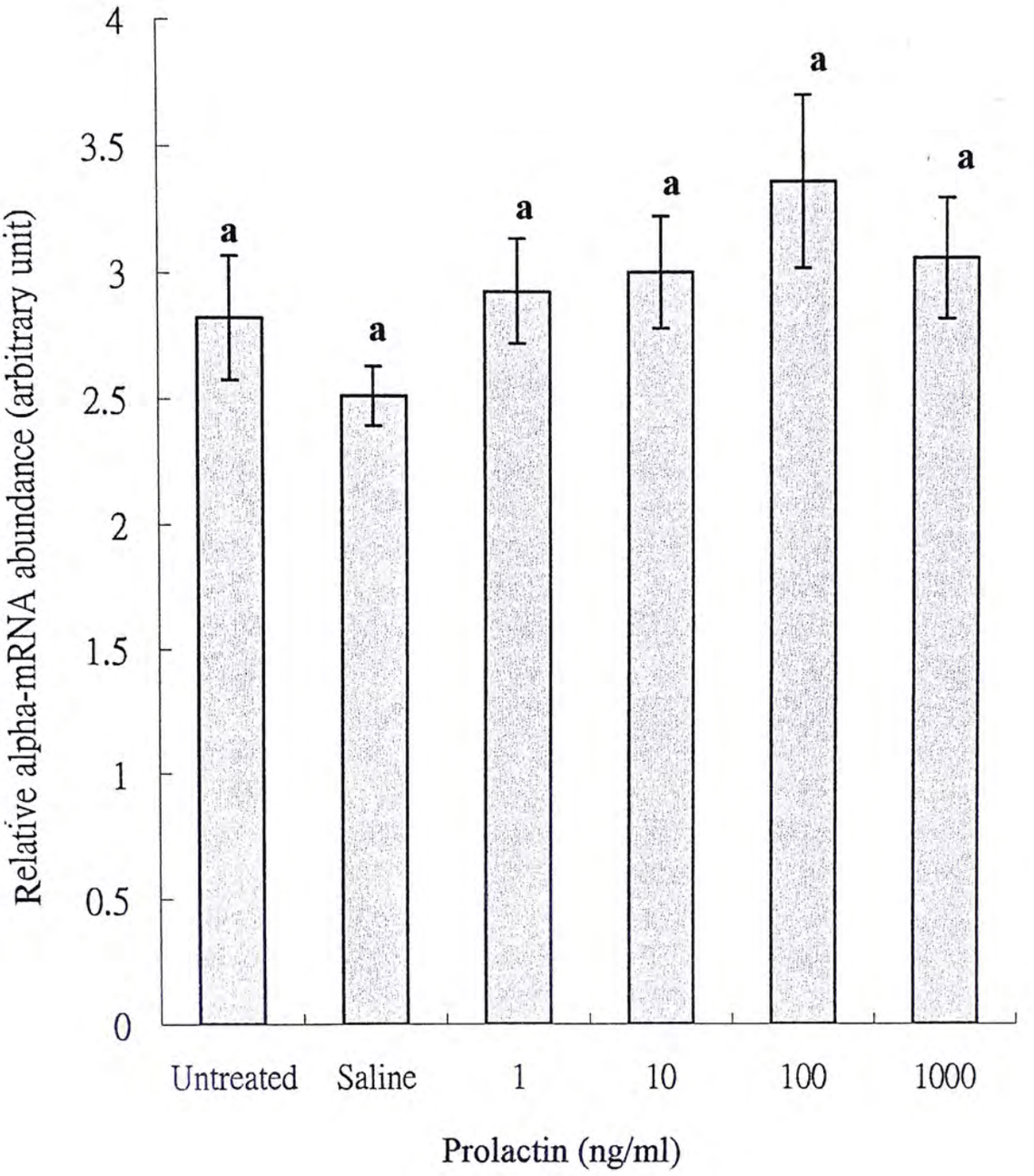


Figure 3.7. *Sparus sarba*: Branchial Na^+-K^+-ATPase α-mRNA after treatment with saline and different doses of ovine prolactin (oPRL) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-mRNA level (n=8). No significant difference (P<0.05) in α-mRNA subunit abundance between the groups. All data were normalized against 18S rRNA.

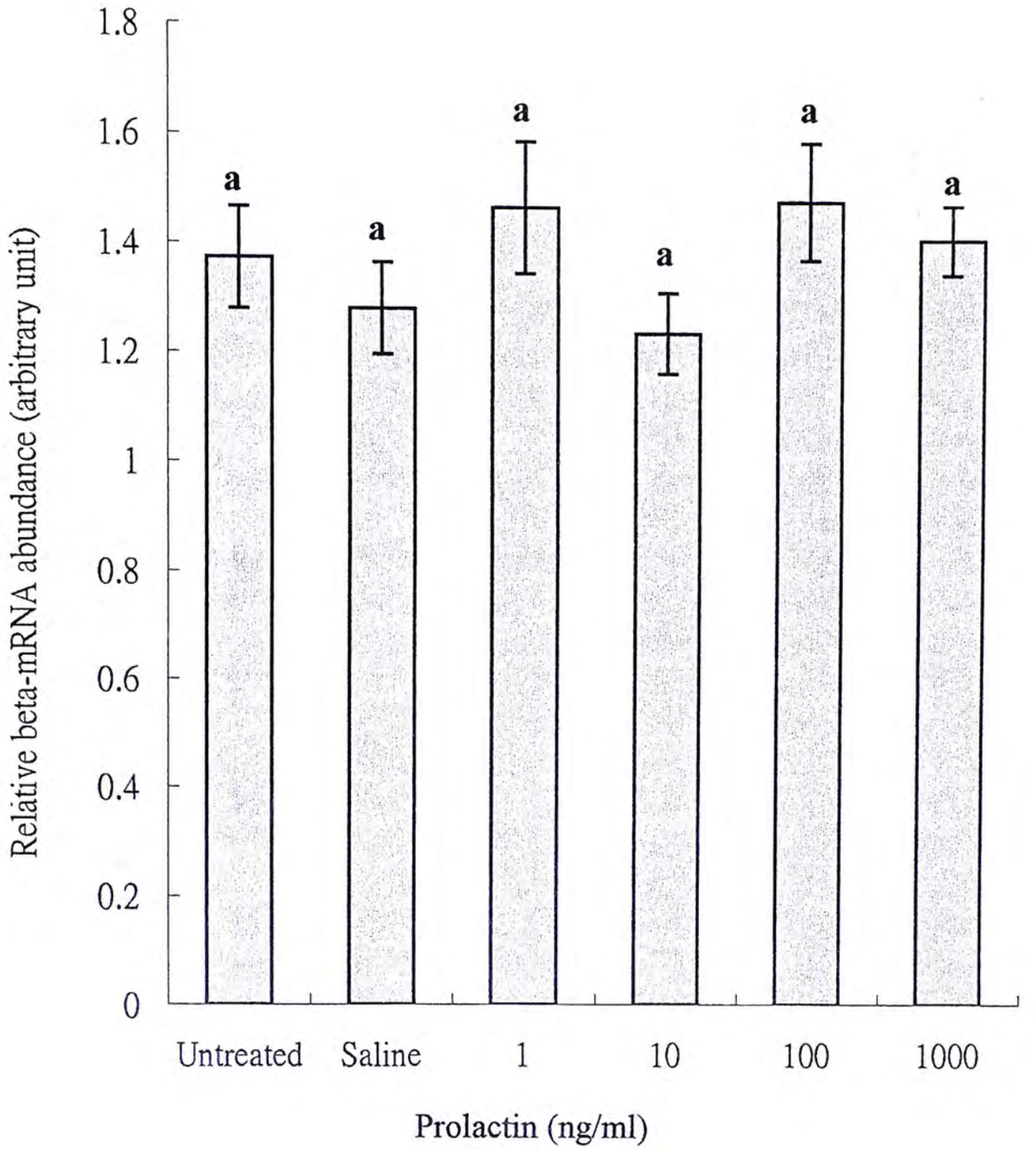


Figure 3.8. *Sparus sarba*: Branchial Na^+-K^+-ATPase β-mRNA after treatment with saline and different doses of ovine prolactin (oPRL) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase β-mRNA level (n=7-8). No significant difference (P<0.05) in β-mRNA subunit abundance between the groups. All data were normalized against 18S rRNA.

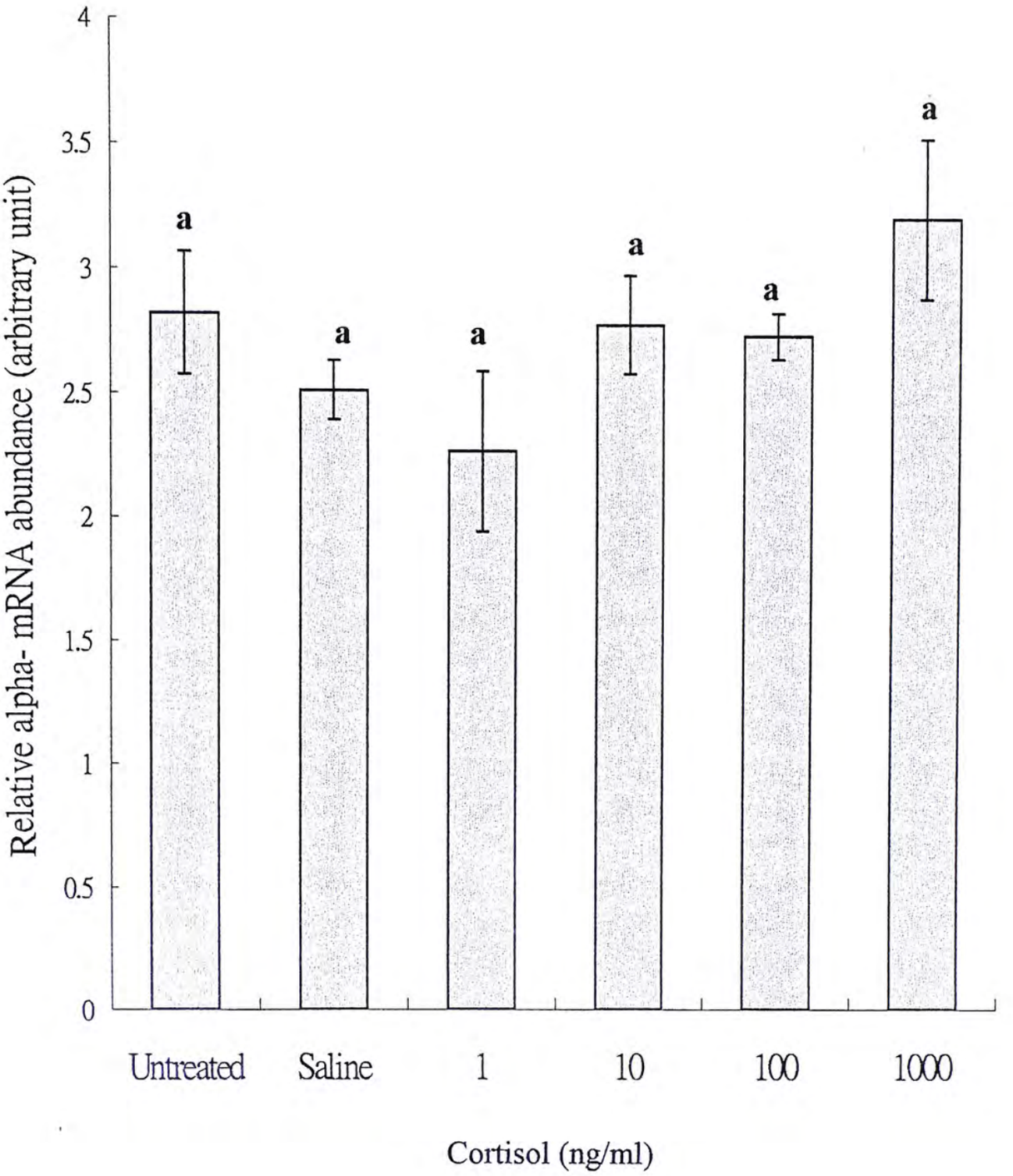


Figure 3.9. *Sparus sarba*: Branchial Na^+-K^+-ATPase α-mRNA after treatment with saline and different doses of cortisol *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-mRNA level (n=7-8). No significant difference (P<0.05) in α-mRNA subunit abundance between the groups. All data were normalized against 18S rRNA.

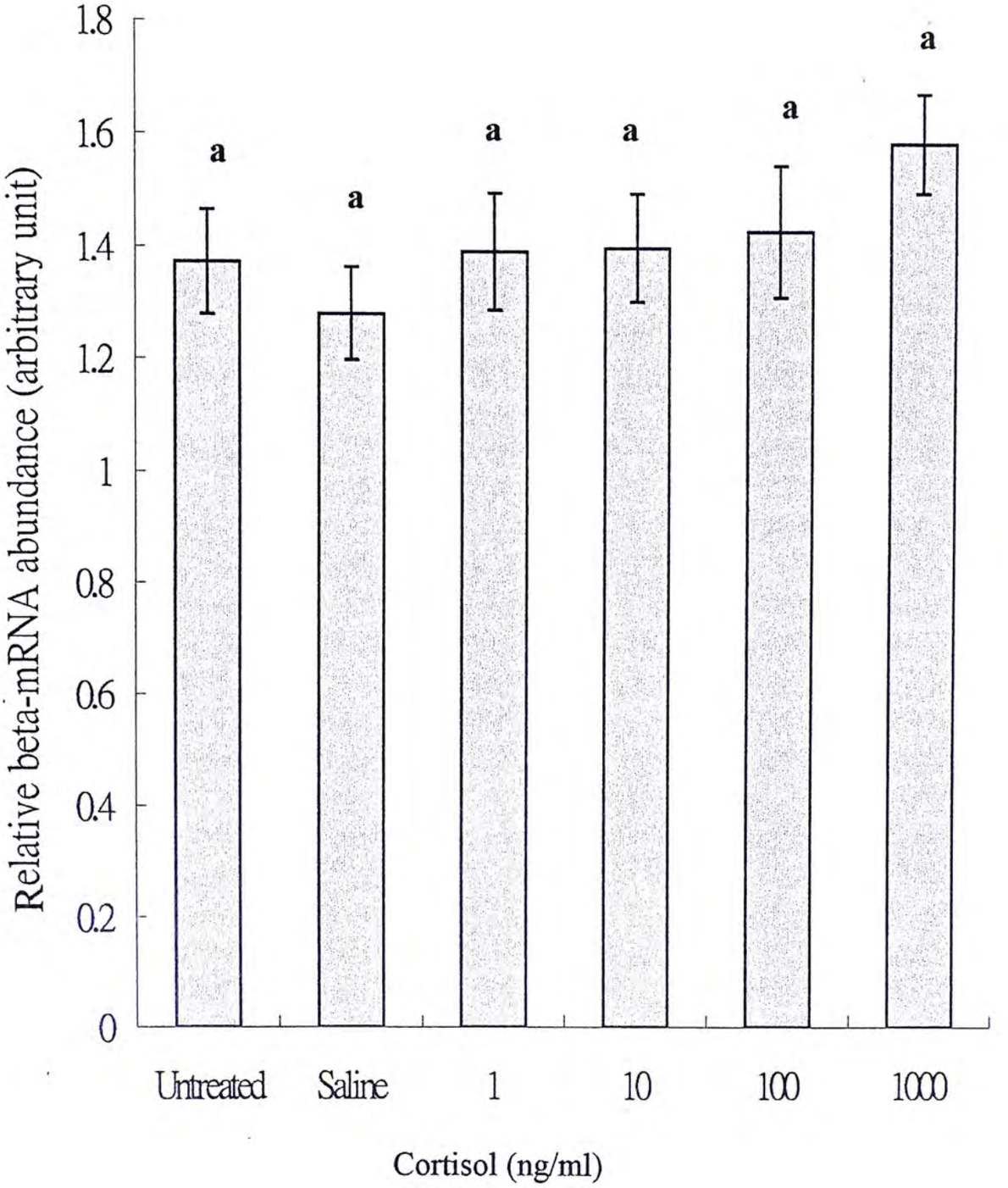


Figure 3.10. *Sparus sarba*: Branchial Na^+-K^+-ATPase β-mRNA after treatment with saline and different doses of cortisol *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase β-mRNA level (n=8). No significant difference (P<0.05) in β-mRNA subunit abundance between the groups. All data were normalized against 18S rRNA.

Table 3.1. The stoichiometric ratio of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunits for gills treated with saline and different doses of hormone in *Sparus sarba*. All values are means ± SEM (n=7-8). *Significantly different from the corresponding saline-treated control group at $P < 0.05$. Values are expressed as percentage change from saline control.

Branchial Na^+-K^+-ATPase α: β mRNA ratio				
Treatment	rbGH	rbIGF-I	oPRL	Cortisol
Saline	100 ± 0.12	100 ± 0.12	100 ± 0.06	100 ± 0.06
1ng/ml	128 ± 0.20	* 200 ± 0.32	103 ± 0.10	100 ± 0.06
10ng/ml	146 ± 0.19	* 189 ± 0.29	116 ± 0.08	100 ± 0.04
100ng/ml	* 193 ± 0.18	* 263 ± 0.28	113 ± 0.05	100 ± 0.06
1000ng/ml	* 191 ± 0.24	* 334 ± 0.58	110 ± 0.11	105 ± 0.11

3.4.2. Analysis of Na^+-K^+-ATPase α-protein subunit

To study Na^+-K^+-ATPase α-protein subunit expression, the analysis yielded an electrophoretic band of 95-100 kDa in all gill samples tested after probed with α-5 antimouse antibodies (Fig. 3.11). rbGH and rbIGF-I treated fish displayed an elevation in α-protein subunit expression compared to saline-treated controls whereas oPRL and cortisol were not significantly altered.

rbGH displayed 2-2.3 fold elevation in Na^+-K^+-ATPase α-protein subunit when 1 and 10 ng/ml rbGH was added but no significantly change at 100 and 1000ng/ml (Figs. 3.12 & 3.13). For rbIGF-I treated fish, it caused near 6.6 fold increase when 1ng/ml of hormone was added. It also caused 3 fold increase at 10ng/ml of hormone but no change in the subunit at higher doses (Figs. 3.14 & 3.15). oPRL (Figs. 3.16 & 3.17) and cortisol (Figs. 3.18 & 3.19) remained relatively unchanged in comparison to saline-treated one.

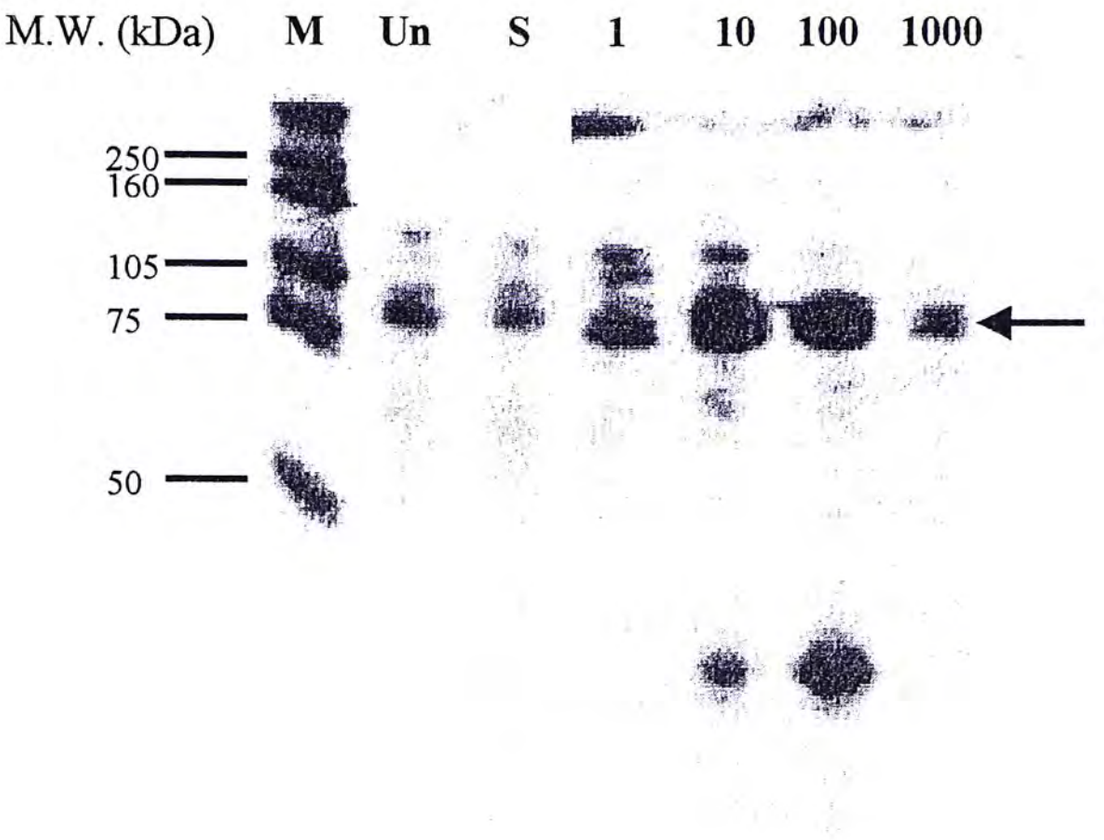


Fig. 3.11. A representative Western blot of branchial protein (10μg) from *Sparus sarba* after treatment with saline (S) and different concentrations of recombinant bream growth hormone (1, 10, 100, 1000ng/ml). The membrane was probed with the primary antibody, monoclonal α-5 antibody which specific to Na^+-K^+-ATPase α-protein subunit. The positions of M and Un indicate protein standard marker and untreated sample respectively. The arrow shows the size around 95-100kDa which immunoreact with α-5 antibody.

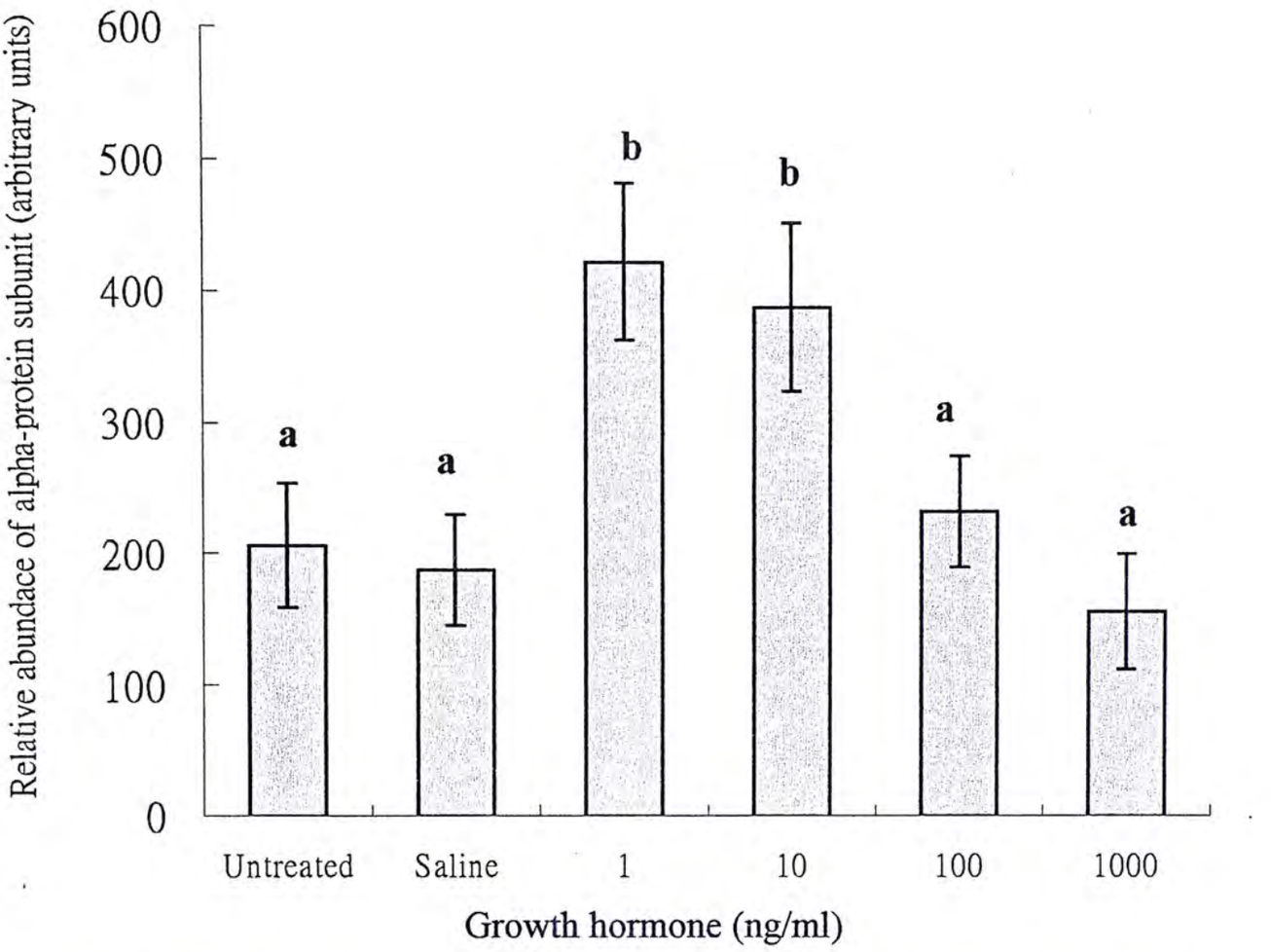


Figure 3.12. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-protein abundance after treatment with saline and different doses of recombinant bream growth hormone (rbGH) *in vitro*. Values are means ± S.E.M. relative branchial Na^+-K^+-ATPase α-protein level compared to saline-treated group (n=6). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

Figure 3.13. Representative of western blot of different doses of rbGH treated *Sparus sarba* branchial tissues *in vitro*. The blot was probed with monoclonal α5 antibody (antimouse) specific to Na^+-K^+-ATPase α-protein subunit.

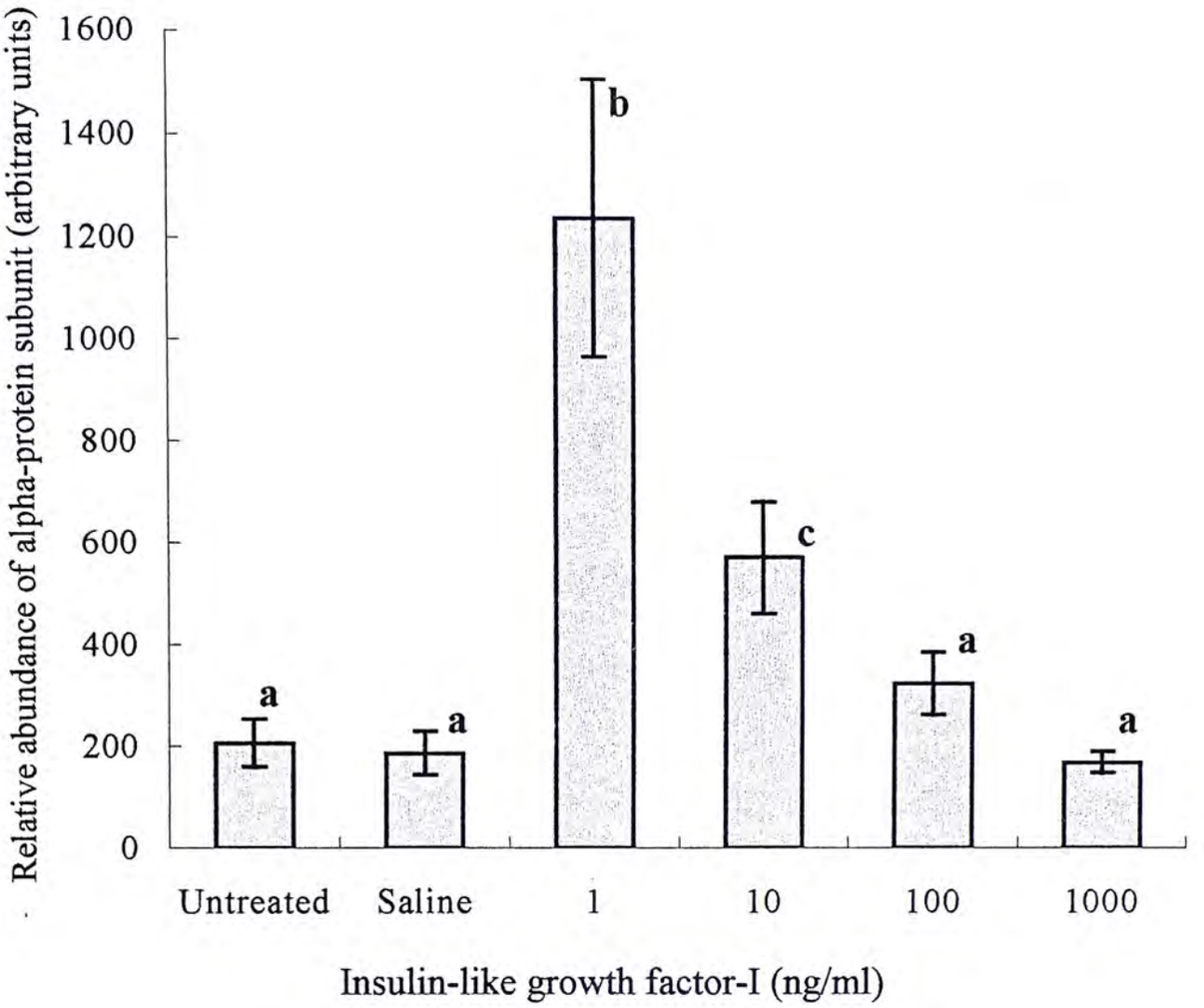


Figure 3.14. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-protein abundance after treatment with saline and different doses of recombinant bream insulin-like growth hormone (rbIGF-I) *in vitro*. Values are means ± S.E.M. relative branchial Na^+-K^+-ATPase α-protein level compared to saline-treated group (n=6). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

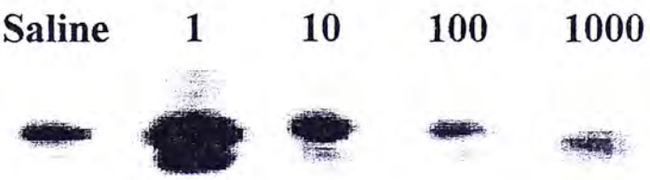


Figure 3.15. Representative of western blot of different doses of rbIGF-I treated *Sparus sarba* branchial tissues *in vitro*. The blot was probed with monoclonal α5 antibody (antimouse) specific to Na^+-K^+-ATPase α-protein subunit.

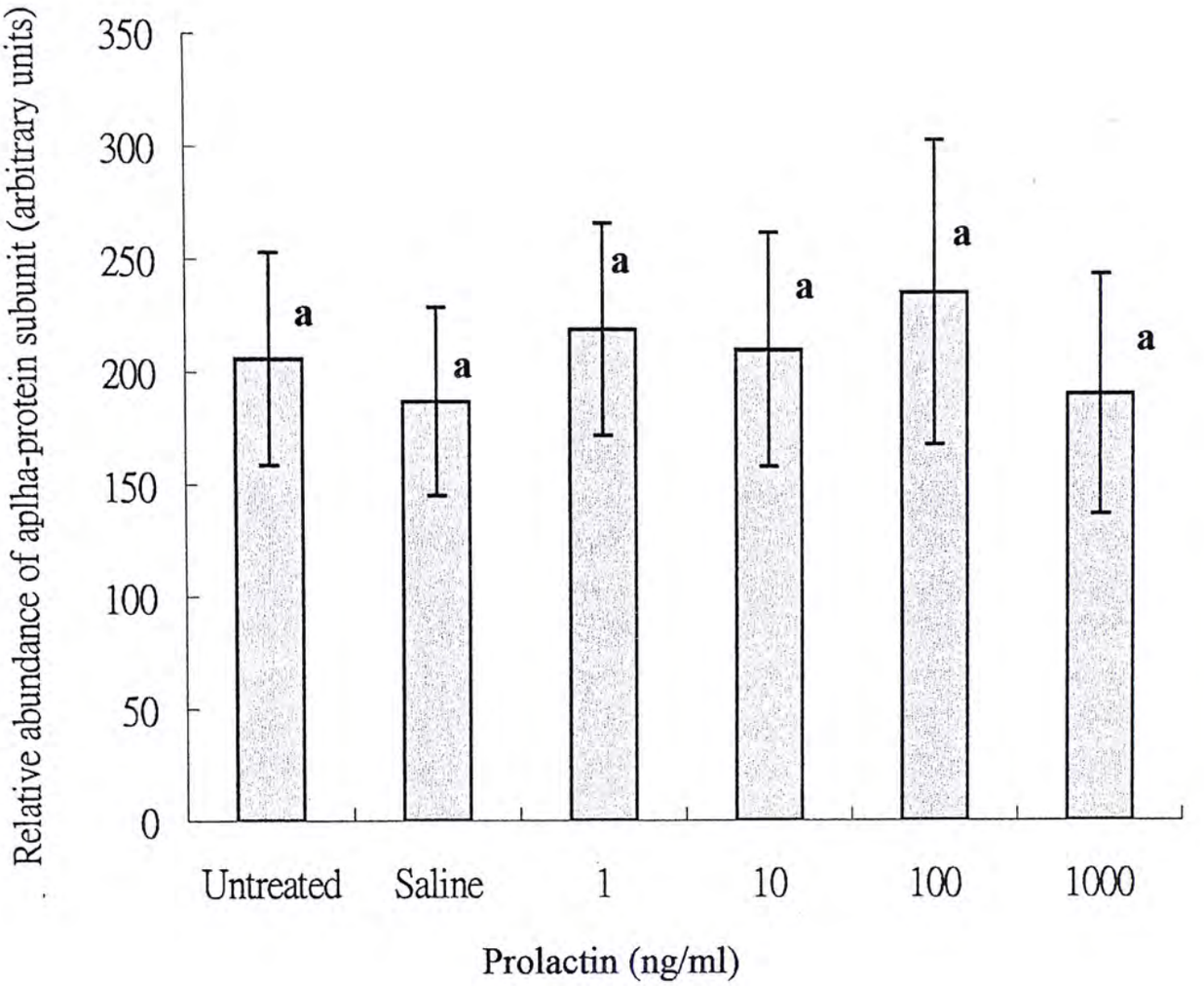


Figure 3.16. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-protein abundance after treatment with saline and different doses of ovine prolactin (oPRL) *in vitro*. Values are means ± S.E.M. relative branchial Na^+-K^+-ATPase α-protein level compared to saline-treated group (n=6). No significant difference (P<0.05) in α-protein subunit abundance between the groups.

Figure 3.17. Representative of western blot of different doses of oPRL treated *Sparus sarba* branchial tissues *in vitro*. The blot was probed with monoclonal α5 antibody (antimouse) specific to Na^+-K^+-ATPase α-protein subunit.

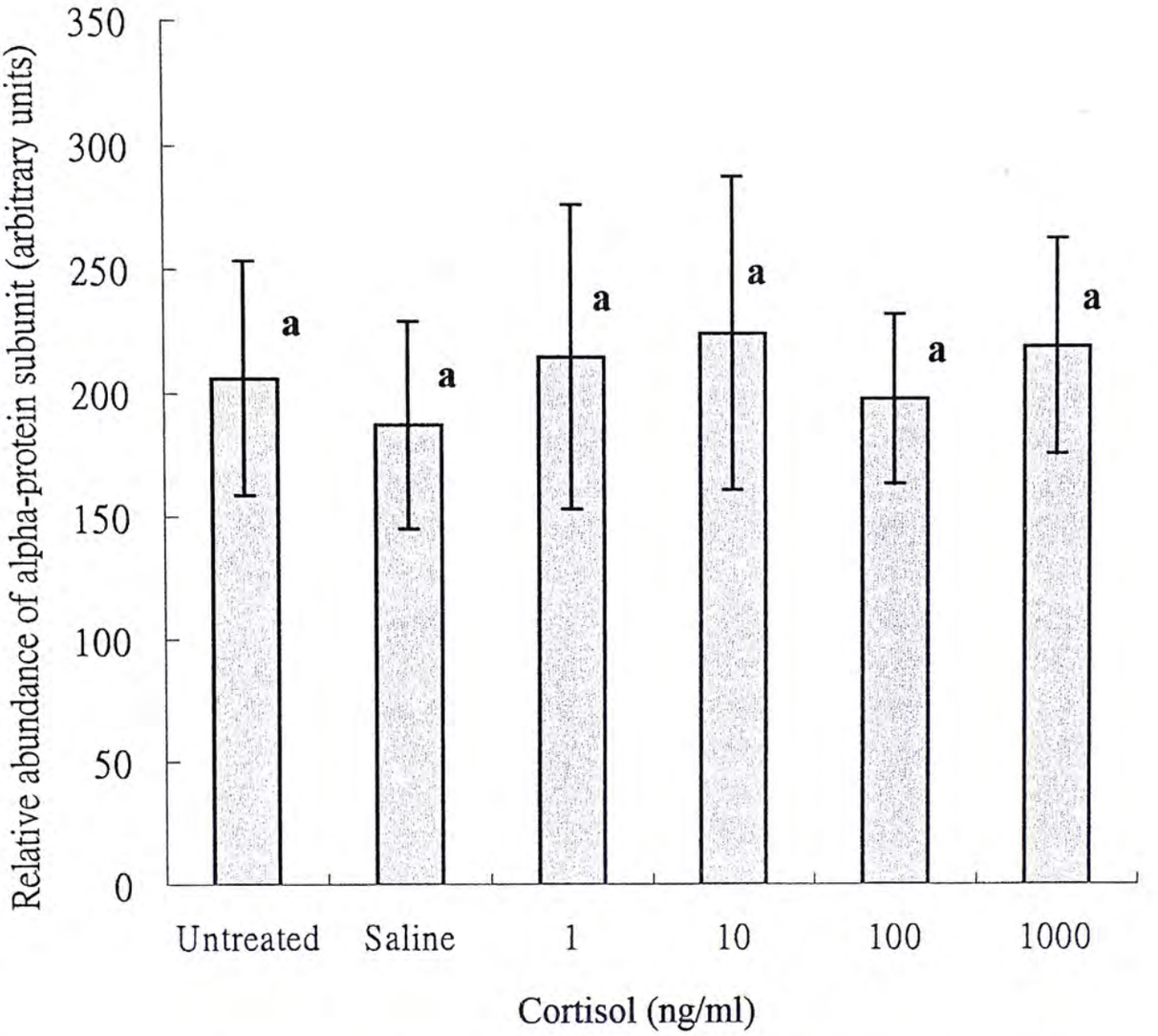


Figure 3.18. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-protein abundance after treatment with saline and different doses of cortisol *in vitro*. Values are means ± S.E.M. relative branchial Na^+-K^+-ATPase α-protein level compared to saline-treated group (n=6). No significant difference (P<0.05) in α-protein subunit abundance between the groups.

Figure 3.19. Representative of western blot of different doses of cortisol treated *Sparus sarba* branchial tissues *in vitro*. The blot was probed with monoclonal α5 antibody (antimouse) specific to Na^+-K^+-ATPase α-protein subunit.

3.4.3. Analysis of Na^+-K^+-ATPase activity

To investigate the hormonal effect on branchial Na^+-K^+-ATPase activity, enzyme assay has been done. Fig. 3.20 shows the effect of different doses of rbGH on branchial Na^+-K^+-ATPase activity. From the results, we observed no significant alterations in functional Na^+-K^+-ATPase activity between untreated and saline-treated fish gills. Different doses of rbGH produced a bell-shape like trend on branchial functional Na^+-K^+-ATPase activity. However, the activity increased significantly only when 10ng/ml rbGH was added, the increase was elevated 1.5 fold when compared with saline-treated control.

For the effect of rbIGF-I on branchial Na^+-K^+-ATPase activity was shown in Fig. 3.21. rbIGF-I elevates the enzyme activity ~1.4 fold at lower dose groups (1, 10ng/ml) significantly but higher dose group (100, 1000ng/ml) had no effect on it.

. oPRL treated gill filament demonstrated a bit lower on branchial Na^+-K^+-ATPase activity in comparison to saline control, however, the effect is not significant (Fig. 3.22).

In Fig. 3.23, it shows a reduction in functional activity after 1ng/ml of cortisol treatment and tends to increase Na^+-K^+-ATPase activity dose dependently, however, the changes are not significant.

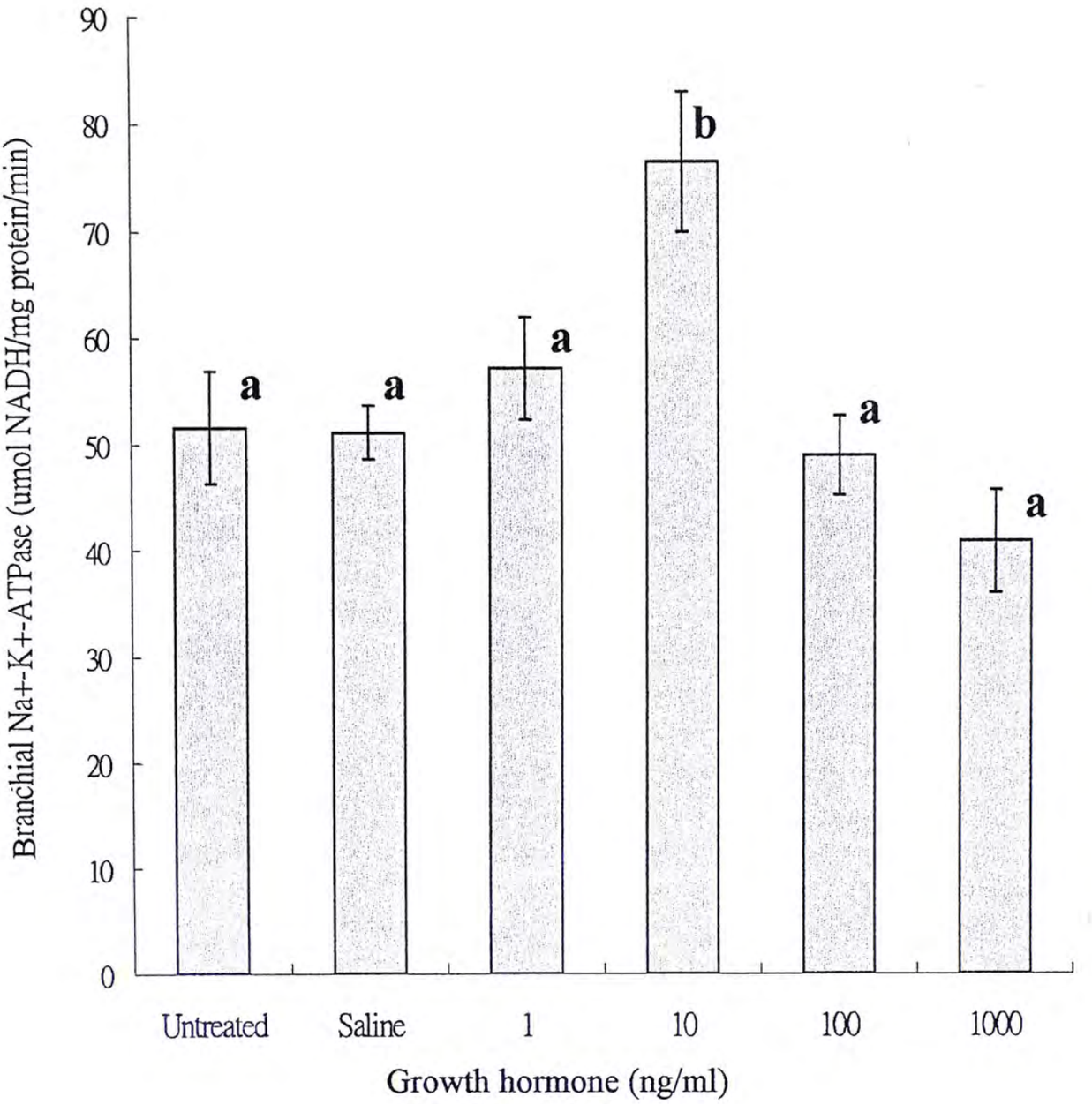


Figure 3.20. *Sparus sarba*: Branchial Na^+-K^+-ATPase activity after treatment with saline and different doses of recombinant bream growth hormone (rbGH) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase activity (n=6). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

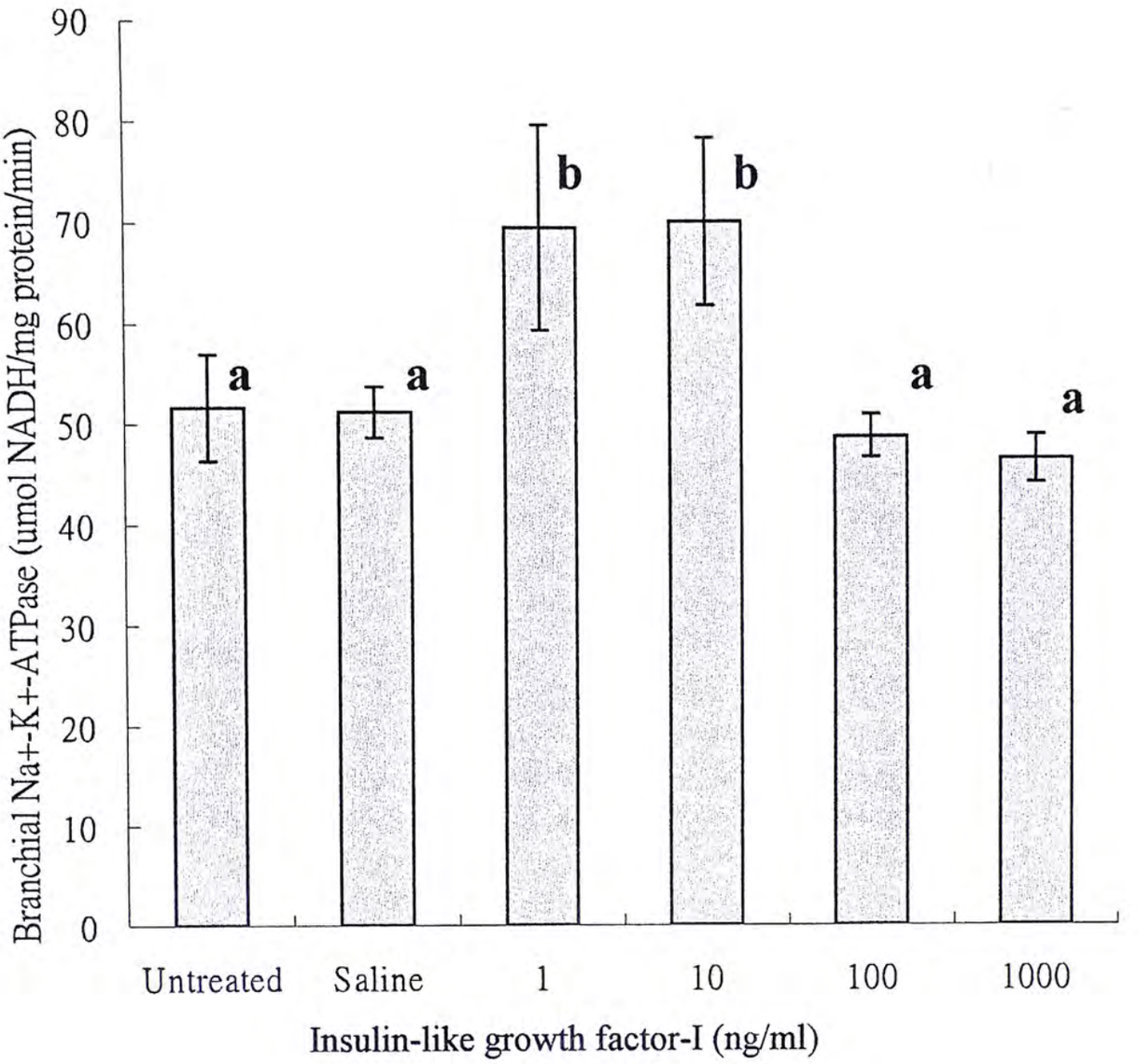


Figure 3.21. *Sparus sarba*: Branchial Na^+-K^+-ATPase activity after treatment with saline and different doses of recombinant bream insulin-like growth factor-I (rbIGF-I) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase activity (n=6). Values with different alphabets are significantly different from each other (P<0.05). Student-Newman-Kuels Multiple Comparison.

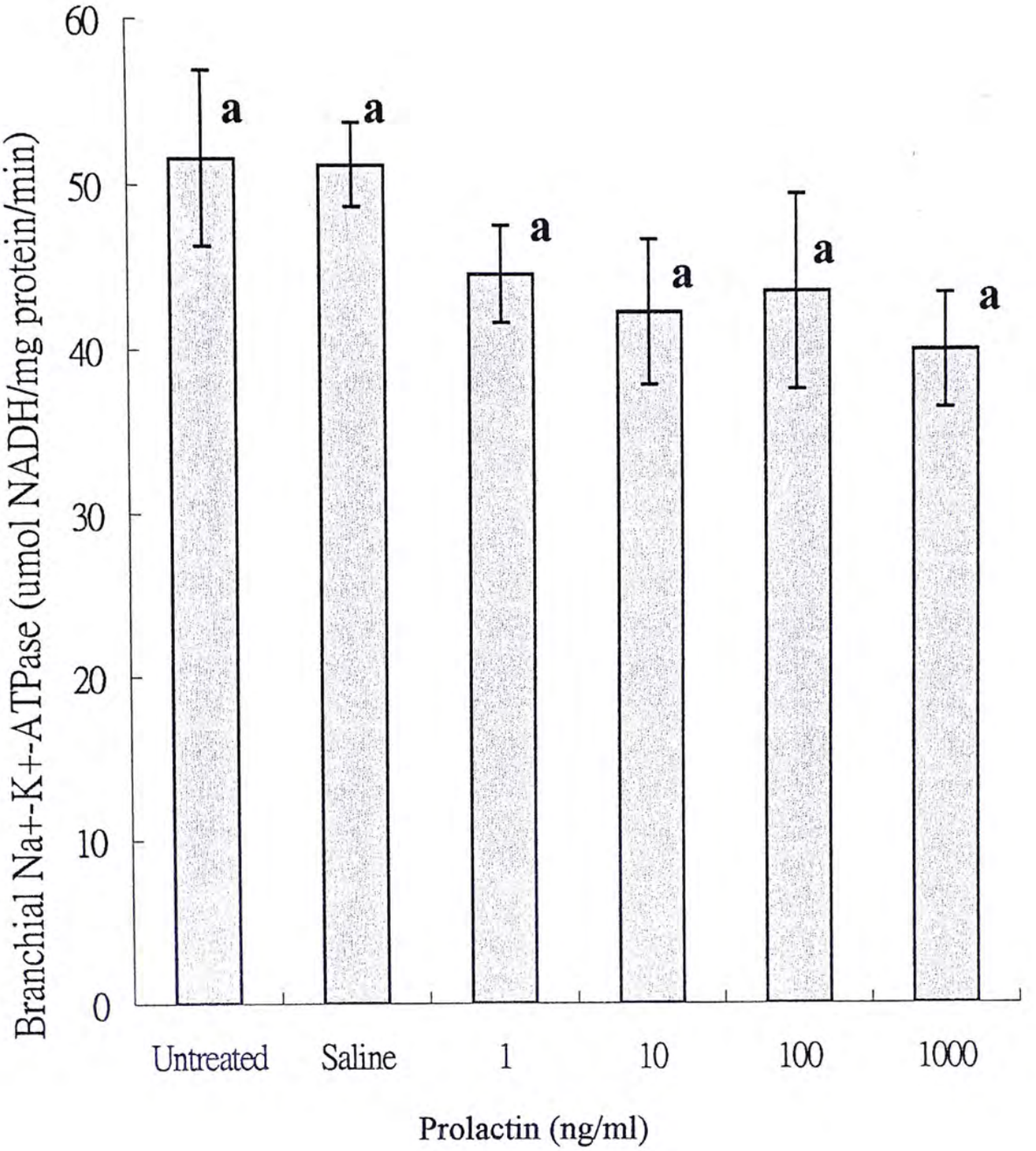


Figure 3.22. *Sparus sarba*: Branchial Na^+-K^+-ATPase activity after treatment with saline and different doses of ovine prolactin (oPRL) *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase activity (n=6). No significant difference (P<0.05) in activity between the groups.

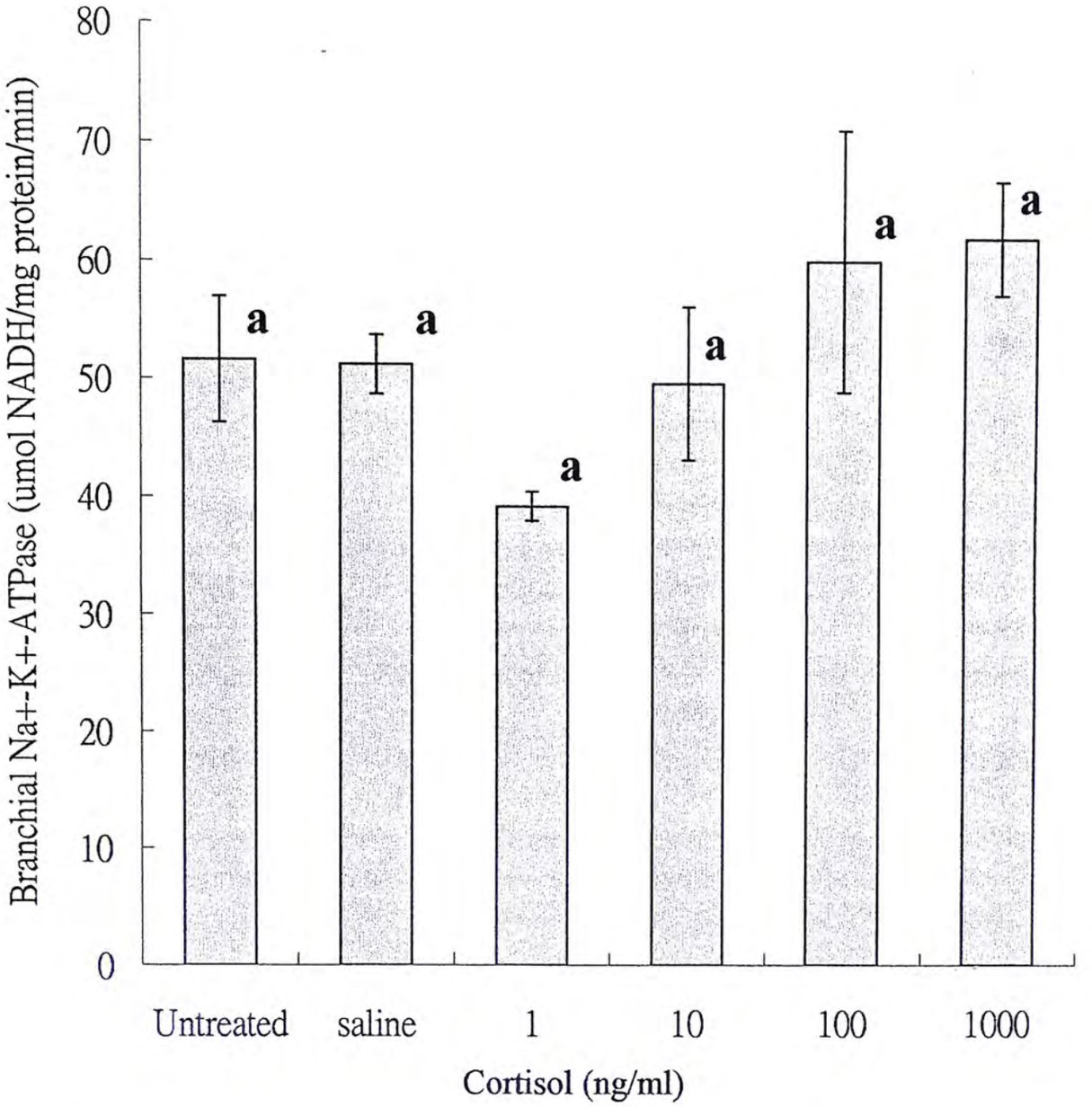


Figure 3.23. *Sparus sarba*: Branchial Na^+-K^+-ATPase activity after treatment with saline and different doses of cortisol *in vitro*. Values are means ± S.E.M. branchial Na^+-K^+-ATPase activity (n=5-6). No significant difference (P<0.05) in activity between the groups.

3.5. Discussion

The hormone concentration from 1ng/ml to 1000ng/ml was used to investigate whether different doses of hormone caused different physiological alteration and regulation of branchial Na^+-K^+-ATPase mRNA and functional activity. By analyzing the abundance of mRNA, protein subunits and the enzyme activity, the effect of hormone on *Sparus sarba* branchial Na^+-K^+-ATPase mechanism and regulation as well as transcription, translation and posttranslational process can be addressed.

In order to achieve a compromise among (1) the threshold time for an observable effect for the subunits, (2) the prevention of the death of gill filament and (3) enough time for the observable responses to be induced, an incubation period of 2hr was adopted in the present investigation. This incubation protocol was in line with the study of D'Cotta *et al.* (2000) in which the abundance of Na^+-K^+-ATPase α-subunit mRNA was observed to rise 3 hr after transferring the fish from freshwater to seawater.

3.5.1. Effects of rbGH and rbIGF-I on Na^+-K^+-ATPase expression

GH has already been described as seawater-adaptating hormone in teleosts. Many studies have described enhanced the hyposmoregulatory ability in salmonids and also in non-salmonids after GH administration. For example, growth hormone treatment in brown trout following transfer from freshwater to seawater enhanced their survival and this response was found to be associated with an increase in the number of the chloride cells and a rise in branchial Na^+-K^+-ATPase levels (Madsen,

1990b). Two species of tilapia, *Oreochromis niloticus* and *Oreochromis mossambicus*, upon seawater adaptation, branchial Na^+-K^+-ATPase expression was also enhanced by growth hormone injection (Sakamoto *et al.*, 1997; Shepherd *et al.*, 1997; Xu *et al.*, 1997). Other than the direct effect of GH on tissue such as elevation of gill Na^+-K^+-ATPase subunits expression, the chloride cells proliferated and displayed extensive morphological differentiation (Sakamoto *et al.* 1993; Madsen *et al.* 1995; McCormick 1996). It also increased primary filament chloride cell density and size (Seidelin *et al.*, 1999). Another possible way for GH to increase seawater tolerance of fish may be in part due to its synergistic effect with cortisol to upregulate gill cortisol receptors (Uchida *et al.*, 1998).

In the marine teleost, *Sparus sarba*, the current study suggests that rbGH elevated branchial Na^+-K^+-ATPase expression in order to increase seawater tolerance. The effect of rbGH on branchial Na^+-K^+-ATPase α- and β-mRNA subunits expression were investigated. There was a 1.7 fold elevation in α-mRNA subunit expression when the gill filament was treated with 1ng/ml rbGH in comparison to saline-treated control group but there was no significant effect on the subunit at higher doses (10, 100, 1000ng/ml rbGH). GH treatment has also been shown to stimulate α-mRNA abundance in brown trout (Madsen *et al.*, 1995). On the other hand, fish treated with 1ng/ml rbGH significantly displayed a 1.9 fold increase in relative β-subunit mRNA abundance but higher doses of rbGH seem to cause a dose dependent reduction in β-subunit mRNA level when compared with saline-treated group, though the reductions are not significant. The results showed that 1ng/ml of rbGH induced transciption in both α- and β-subunit mRNA. The increase in relative mRNA abundance may be due to higher subunits gene expression, stimulation of

transcriptional regulation and/or lower degradation rates of the existing mRNA, all of which means increasing the stabilization or rapid turnover of mRNA subunits. The result here *in vitro* was different from an *in vivo* study on the same species which was done by Deane *et al.* (1999). Injection of rbGH into *Sparus sarba* did not cause any significant alteration in both Na^+-K^+-ATPase α- and β-subunit mRNA. This indicated that rbGH can act directly on gill Na^+-K^+-ATPase to stimulate mRNA expression but the effect may be mimicked by other factors *in vivo.* These observations further suggest that low dose of rbGH has an induction effect on both silver seabream gill Na^+-K^+-ATPase α- and β-mRNA subunits *in vitro.*

There are different isoforms of Na^+-K^+-ATPase α- and β-subunits and heterogeneity of the α-subunit with various isoforms in higher vertebrates has been demonstrated (Shamraj and Lingrel, 1994; Shull *et al.*, 1985). These isoforms show different affinities for Na^+ and K^+, with varying sensitivity to the inhibitor ouabain. The presence of three different α-mRNA transcripts of 3.3, 3.7 and 5kb, with an homogeneous α-cDNA in wild masu salmon *Oncorhynchus masou* (Ura *et al*, 1997). Only α1- and β1- mRNA subunits were detected by using α1- and β1- cDNA probes in this study. Effect of different doses of hormone on other mRNA subunit isoforms expression cannot be investigated. The present study did not produce evidence for the existence of multiple functional α-isoform transcripts in the gill tissue. Furthermore, it is also possible that more than one α-isoform mRNA and more than one β-isoform mRNA are confined to the 3.3kb and 2.4kb size band respectively, and different isoforms could be comigrating due to similarities in their size. Northern blot analysis were revealed α-subunit transcript with 3.3kb in size and β-subunit transcript 2.4kb in the species *Sparus sarba* (Deane *et al.*, 2000).

Na^+-K^+-ATPase enzyme consists of two polypeptide subunits, the catalytic α-subunit consists of 1022 amino acids and has a calculated molecular weight of about 100 to 112 kDa and a β-subunit which about 55kDa in weight (Kawakami *et al.*, 1985; Geering 1990). To test for the effect of different concentration of hormone on Na^+-K^+-ATPase α-protein subunit, monoclonal α-5 antibody which is specific to Na^+-K^+-ATPase α-protein subunit was used. There may not be too specific because of multiple bands were shown on the blot. Na^+-K^+-ATPase α-protein subunit is likely the immunoreactive band about 95-100 kDa in size which is of the expected size as reported by Kawakami *et al.* (1985) and Geering (1990). There are some ways to ensure the immunoreactive band is Na^+-K^+-ATPase α-protein subunit. The bands can be checked by using homogenous *Sparus sarba* Na^+-K^+-ATPase α-protein subunit antibody or through the amino acid analysis of the bands.

rbGH stimulated α-protein abundance when 1, 10 ng/ml rbGH was added but no significantly change occurred at 100 and 1000ng/ml. Rise in protein level may be due to increased stability of existing mRNA subunit to translate into protein subunit and/or increased the translation process to produce more protein subunit directly and/or higher percentage of α-protein matures at the time. Whether one or two or all of these parameters leads to the rise in α-protein detected is not clear.

In the current study, attempts have been made to evaluate β-protein levels with five different antibodies, but all resulted in no specific binding so that the effect of hormones on β-protein subunit expression, could not be ascertained.

Comparing the effects of rbGH on mRNA subunits and α-protein subunit expression, the data showed that rbGH elevated branchial Na^+-K^+-ATPase α-mRNA and β-mRNA subunit at dose of 1ng/ml but provoked α-protein subunit at 1, 10 or 100ng/ml hormone treatment. rbGH induces Na^+-K^+-ATPase transciption at lower dose only (1ng/ml) but induces the translational process in a wider dose range (1-100ng/ml hormone treatment). Moreover, the result revealing rbGH also promoted the transcriptional process of Na^+-K^+-ATPase β-subunit but only at 1ng/ml hormone treatment.

Increase in gill Na^+-K^+-ATPase activity after 10ng/ml rbGH treatment may be due to the result of higher pumping activity and/or an increase in enzyme synthesis indicating posttranslation. Some studies reported that GH increased total chloride cell number and size in the primary gill filament of salmonids (Richman and Zaugg, 1987a,b; Madsen, 1990b). Due to the fact that Na^+-K^+-ATPase is highly expressed in the basolateral tubular system of the chloride cell, the number of chloride cells is positively related with Na^+-K^+-ATPase activity.

Treatment with higher doses of rbGH (100 and 1000ng/ml) significantly elevated the stoichiometric ratio of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunit abundance, indicating α-mRNA subunit is more sensitive to rbGH or the induction of rbGH to the subunit expression is higher than that of β-mRNA subunit. Comparing the effects of rbGH on mRNA subunits and functional activity, significant high ratio of α-mRNA to β-mRNA subunit abundance (treated with 100 or 1000ng/ml rbGH) resulted in no elevation of enzyme activity.

Upregulation of α-mRNA alone is not physiologically relevant. Although we know that α-subunit carries the catalytic and ion transport properties, β-subunit appears to be necessary for modulating protein maturation and enzyme translocation as well as stabilizing the α-subunit. β-subunit is therefore a requirement and is a rate determining factor for a functional Na^+-K^+-ATPase pump. No matter how high the level of α-mRNA is, it will not be translated into functional enzyme if the β-subunit is not concomitantly upregulated. Complete processing of subunit assembly is essential for the functioning of the enzyme. This event can be seen from the relative ratio of α-mRNA to β-mRNA abundance. If the ratio is near to 1:1, which is the case in the saline-treated controls, the gene expression of both subunits is more or less the same to produce complementary α- and β-protein subunits for α-β complex formation, then the number of functional hydrolytic Na^+-K^+-ATPase units increased in proportion to increases in Na^+-K^+-ATPase activity. On the other hand, higher doses (100, 1000ng/ml) of rbGH increased the ratio of α-mRNA to β-mRNA abundance, tending to decrease functional Na^+-K^+-ATPase activity.

The results would suggest a three-stage modulation mechanism of Na^+-K^+-ATPase. The first modulation possibly acts on the α-gene expression seen at the transcriptional level, the second regulation acts at the translation process and the third stage regulation acts posttranscriptionally. The current study indicated the effect of rbGH on branchial Na^+-K^+-ATPase acts via the transcriptional, translational and posttranslational processes separately. rbGH increased transcription of both mRNA subunits significantly at a concentration of 1ng/ml, induces the translational process in a wider range (1 and 10ng/ml) and increased posttranslational process at 10ng/ml. The threshold concentration of rbGH is therefore different for the induction effect on

α-and β-mRNA, α-protein and enzyme activity and mainly affected at the physiological level of GH (1 to 10ng/ml) but not at higher level of GH (100 and 1000 ng/ml) which are not physiologically relevant.

Different rates of α-mRNA transcription and translation have been detected in mammals, not only for the α-subunit but also for the β-subunit (Azuma *et al.*, 1993). In muscle cells, a 5-fold increase was found for α_2-mRNA after 3,5,3'-triiodothyronine injection, but only a 3-fold rise was found for α-protein. In contrast, the β-subunit mRNA increased nearly 4-fold, and its protein increased 2-fold, whereas the activity was estimated to be only 0.5-fold higher and Na^+-K^+-ATPase activity is also regulated by the β-subunit, since the β-protein influences the stability of the α-polypeptides (Geering, 1991). Based on studies on the time course of Na^+-K^+-ATPase responses in parr-smolt transformation in Atlantic salmon, D'Cotta *et al.* (2000) reported that stimulation of Na^+-K^+-ATPase activity depends first on an increase in the α-mRNA expression which is followed by a slower rise in α-protein abundance that eventually leads to a higher synthesis of Na^+-K^+-ATPase.

In addition, one possible reason for the effect of rbGH induced branchial expression is that it induced paracine IGF-I production. Recombinant bovine growth hormone injected channel catfish showed a higher plasma IGF-I levels when compared with saline-injected controls (Silverstein *et al.*, 2000). Elevation of IGF-I plasma levels by GH treatment has been shown in a variety of vertebrates including teleosts (Humbel, 1990; Moriyama, 1995). The result observed may be due to whether rbGH is acting directly on the tissue and/or mediated and induced by the paracrine induced IGF-I or the combination of both rbGH and IGF-I actions.

IGF-I treatment in freshwater fish (*Salmo trutta*) increases hypoosmoregulatory ability. On the other hand, the GH/IGF-I axis plays an important role in the seawater adaptation of salmonids and non-salmonids (Sakamoto and Hirano, 1993; Sakamoto *et al.*, 1993, 1997; McCormick, 1995). More recently, Mancera and McCormick (1999) found that either ovine GH or bovine IGF-I injections increased salinity tolerance in the killfish, *Fundulus heteroclitus*. In rainbow trout and Altantic salmon, the injection of bovine IGF-I enhances the ability of the fish to osmoregulate when they are transferred from freshwater to brackish water, however, the effect was not as great as that of growth hormone (McCormick *et al.*, 1991; McCormick, 1996).

The present data showed that rbIGF-I elevated branchial Na^+-K^+-ATPase α-mRNA subunit in all doses (1.5-2.5 fold increase) but significantly reduced β-mRNA subunit in a dose dependent manner. Regarding a rbIGF-I effect on α-protein levels, a near 4 fold increase was observed when 1ng/ml of hormone was added and significant rise in protein level was also seen when gills were treated at a dose of 10ng/ml. Comparing the effects of rbIGF-I on mRNA subunits and α-protein subunit expression, rbIGF-I induced branchial Na^+-K^+-ATPase α-mRNA subunit transciption at all doses investigated but only induced the translational process at lower doses (1, 10ng/ml). Moreover, the result revealing rbIGF-I inhibits the transcriptional process of Na^+-K^+-ATPase β-subunit.

Increase in the number of functional Na^+-K^+-ATPase pumps depends upon the upregulation of both α- and β-subunits. Thus, higher ratio of relative Na^+-K^+-ATPase α-mRNA: β-mRNA causes no elevation in Na^+-K^+-ATPase activity (at higher doses

of rbIGF-I). For the effect of rbIGF-I on branchial Na^+-K^+-ATPase activity, 1 and 10ng/ml rbIGF-I elevated the enzyme activity significantly but higher doses had no effect on it. Similar effects were displayed *in vivo* in other species. rbIGF-I injection were observed to stimulate gill Na^+-K^+-ATPase αmRNA levels and enzyme activity in freshwater acclimated *Salmo trutta* (Madsen *et al.*, 1995) and in 10ppt seawater-acclimated killifish, *Fundulus heteroclitus* (Mancera and McCormick, 1998, 1999).

Treatment with all doses of rbIGF-I (1, 10, 100 and 1000ng/ml) significantly elevated the stoichiometric ratio of branchial Na^+-K^+-ATPase α-mRNA to β-mRNA subunit abundance, indicating α-mRNA subunit is more sensitive to rbIGF-I than β-mRNA subunit. Comparing the effects of rbIGF-I on mRNA subunits and functional activity, exceedingly high ratio of α-mRNA to β-mRNA subunit abundance (treated with 100 or 1000ng/ml rbIGF-I) did not elevate functional activity because of upregulation of β-subunit expression is also needed for the synthesis of a functional Na^+-K^+ pump.

rbIGF-I increased the transcription process of α-subunit at all doses but decreased β-subunit transciption at high doses (10, 100 or 1000ng/ml) significantly. It induces the α-subunit translational process at 1 or 10ng/ml and increased posttranslational process at 10ng/ml.

The present study demonstrated that after both rbGH and rbIGF-I treatment, there was a higher fold of increase in mRNA subunit abundance than that in enzyme activity. These data suggested that the branchial cells respond more sensitively to mRNA level increase than in enzyme level and/or the increase in subunits gene

expression may not be the only mediator of enzyme activity. Hormonal regulation may not occur only at the transcriptional level but also at translational and posttranslational levels. Na^+-K^+-ATPase is a multisubunit enzyme and becomes functional only when the α-β complex is formed. An excess or over synthesis of Na^+-K^+-ATPase α-subunit mRNA may not be physiologically relevant because the α-subunit may be susceptible to proteolytic degradation if it does not combine with the β-subunit. Geering *et al* (1989), by studying *Xenopus* oocytes, suggested that if the β-subunit level is much lower than that of α-subunit, the uncomplexed α-subunit is highly trypsin sensitive and will undergo proteolytic degradation. The structural stability of endogenous α-subunit and number of functional Na^+-K^+-ATPase units were elevated when β-mRNA subunits were microinjected into these oocytes.

Na^+-K^+-ATPase is located on the basolateral membrane of chloride cells and each chloride cell has more than 10^8 Na^+-K^+-ATPase moles, therefore, an increase in branchial chloride cell number is normally associated with a parallel increase in Na^+-K^+-ATPase activity (Avella *et al.*, 1993; Kultz *et al.*, 1992). Seidelin *et al.* (1999) reported that IGF-I injection increased Na^+-K^+-ATPase immunoreactive cell number in the primary gill filament. The effect of rbIGF-I may thus be associated with a combination of chloride cell stimulation leading to increase in Na^+-K^+-ATPase activity and direct action on Na^+-K^+-ATPase expression at lower dose (1, 10ng/ml) when IGF-I was added to the gill filament.

Moreover, the osmoregulationary functions of IGF-I may depend on interactions with endogenous hormonal factors. IGF-I and cortisol had additive stimulatory effects on Na^+-K^+-ATPase activity and α-subunit Na^+-K^+-ATPase mRNA

level in the gills and increased the number of Na^+-K^+-ATPase-immunoreactive cells in the primary filament (Seidelin *et al.*, 1999; McCormick, 1996). The observed IGF-I effects in this study may be due to the additive effect of IGF-I with existing cortisol on the gill filament. Madsen and Bern (1993) showed that *in vivo* GH treatment may increase the sensitivity of *Oncorhynchus kisutch* gill Na^+-K^+-ATPase to subsequent stimulation with IGF-I *in vitro*. When GH is combined with IGF-I, the two hormones stimulate gill Na^+-K^+-ATPase activity and α-mRNA content additively (Seidelin and Madsen, 1997). Many of the actions of growth hormone are mediated by IGF-I. Thus, endogenous or existing GH initiates rbIGF-I functioning on Na^+-K^+-ATPase expression and rbIGF-I may act with the endogenous GH and causes significant changes in subunits expression and enzyme activity.

3.5.2. Effects of oPRL on Na^+-K^+-ATPase expression

From the results, all doses of oPRL had no significant effect on both *Sparus sarba* Na^+-K^+-ATPase mRNA expression and functional enzyme activity *in vitro*.

Concerning the commonly accepted role of PRL in fish osmoregulation, ion uptake is promoted in both euryhaline and stenohaline fish in freshwater. When fishes in seawater are treated with PRL, ion excretion, primarily from the gill, is inhibited, resulting in increased plasma ion concentrations and osmolality (Hirano, 1986; Brown and Brown, 1987; Madsen and Bern, 1992). In hypophysectomized black bullhead oPRL restored plasma Na^+ and plasma osmolarity to the level of intact fish and similar effects also have been observed in other species such as hypophysectomized

Indian catfish and channel catfish *Ictalurus punctatus* (Chidambaram *et al.*, 1972; Parwez and Goswami, 1985; Eckert *et al.*, 2001).

Functional studies of fish PRL were carried out using heterologous PRLs and the results varied greatly between experiments and species. There is no clear consensus with respect to the effects of PRL on gill Na^+-K^+-ATPase. Atlantic salmon treated with PRL implants exhibited an increase in Na^+-K^+-ATPase activity (Boeuf *et al.*, 1994) but *in vitro* exposure of coho salmon gill tissue to PRL had no effect on Na^+-K^+-ATPase activity (McCormick *et al*, 1991). Other studies have shown that PRL either decreases (Sakamoto *et al.*, 1997; Shepherd *et al.*, 1997; Kelly *et al.*, 1999c), increases (Boeuf *et al.*, 1994; Leena and Oommen, 2000) or has no effect (Madsen *et al.*, 1995; Eckert *et al.*, 2001) on Na^+-K^+-ATPase activity in the gill. In some cases this variability was due to the ability of these PRLs to bind to both growth hormone and PRL receptors and may be also related to the use of heterologous hormones, species variability, or developmental differences (McCormick, 1995). It appears that the actions of PRL cannot be generalized for all fish due to marked differences between species.

Administration of ovine prolactin to silver seabream (*Sparus sarba*) *in vivo* (Deane *et al.*, 1999) significantly reduced α-subunit mRNA levels by approximately 0.5 fold but had no effect on β subunit mRNA levels. Conversely, α-subunit mRNA levels were not affected by PRL in the brown trout (*Salmo trutta*) (Seidelin and Madsen, 1999). To fully elucidate the effects of PRL on gill Na^+-K^+-ATPase, studies should be carried out on different species of fish using homologous hormones.

The present observation that Na^+-K^+-ATPase expression is not altered by oPRL suggests that the oPRL-induced alterations in the osmoregulatory physiology of fish did not involve modulation of the principal ionomotive enzyme of the fish gills. These results are consistent with the observations of McCormick *et al.* (1991) who found that *in vitro* exposure of coho salmon gill tissue to PRL had no significant effect on Na^+-K^+-ATPase activity. There are two probable reasons for this lack of effect on the ion-transporter expression. Firstly, the general role of PRL is thought to involve a reduction in passive ion and water permeability and not altered ion uptake (Hirano, 1986; Bern and Madsen, 1992). Secondly, many of the actions of PRL in teleost fish have been suggested to be permissive, requiring the presence of other hormones, especially cortisol (Hirano and Mayer-Gostan, 1978). For example, in cultured gill epithelia, cortisol has been demonstrated to increase epithelial "tightness" principally by reducing paracellular permeability, presumably via the formation of tight juctions. This explains the ability of PRL to increase net Na^+ absorption in the presence of cortisol in these kinds of preparations. This possibility can be arrived when comparing the effects of PRL with or without cortisol treatment.

3.5.3. Effects of cortisol on Na^+-K^+-ATPase expression

Cortisol plays an important role in the hydromineral balance of fish. Cortisol has been identified largely as a seawater-adapting hormone in many species, primarily stimulating ion excretion at the gills. Stimultaneous administration of cortisol markedly increased urinary sodium excretion. In branchial tissue, the mechanisms of action include alterations in gill chloride cell morphology and development and regulation of key ion-transporting enzymes, all of which generally result in increased

salinity tolerance during seawater entry (McCormick, 1995). This hormone increases the surface area of gill chloride cells, promotes the functional differentiation of chloride-secreting cells, stimulates gill Na^+-K^+-ATPase activity, and Na^+-K^+-ATPase α-subunit expression, and increases salinity tolerance in numerous teleost species (Foskett *et al,*. 1981; Dange, 1986; Madsen, 1990; Perry *et al.*, 1992; Madsen *et al.*, 1995; McCormick, 1995).

In 1998, Hwang *et al.* implied that transciption and translation of the Na^+-K^+-ATPase α-subunit were enhanced after cortisol treatment in *Oreochromis mossambicus*. *In vivo* study of cortisol administration on branchial Na^+-K^+-ATPase in silver seabream *Sparus sarba* was demonstrated by Deane *et al.* (1999, 2000). The studies showed that cortisol induced 1.4-1.9 fold elevation both in Na^+-K^+-ATPase α- and β-subunit mRNA relative to saline-treated control. The ratio of α: β mRNA also increased. They suggested that cortisol increased Na^+-K^+-ATPase α- subunit gene expression or subunit mRNA stabilization or turnover. However, in the current *in vitro* study, different doses of cortisol had no significant effect on branchial Na^+-K^+-ATPase mRNA subunits in *Sparus sarba in vitro.* As reported by Deane *et al.* (2000), the branchial α: β mRNA subunit ratio was doubled in *Sparus sarba* after cortisol administration *in vivo* in comparison to saline-treated group. However, the present *in vitro* study failed to observe any change in the ratio of cortisol-treated gills. Different results are therefore obtained in the same species with *in vitro* and *in vivo* experiments. It appears that the physiological changes induced by cortisol may not involve modulation of branchial Na^+-K^+-ATPase, and other factors may be required for mediating cortisol-induced Na^+-K^+-ATPase regulation. The actions may be not directly but may be through others indirectly.

Other than its effect on branchial Na^+-K^+-ATPase, an effect of cortisol on intestinal Na^+-K^+-ATPase has also been investigated. The intestinal Na^+-K^+-ATPase activity increased after cortisol injection *in vivo* (Madsen, 1990a,b) but cortisol prevented the decrease in Na^+-K^+-ATPase *in vitro* (Veillette and Young, 1997) during SW acclimation in salmon. This experiment underlined that different responses to cortisol can be found between *in vivo* and *in vitro* studies on the same tissue.

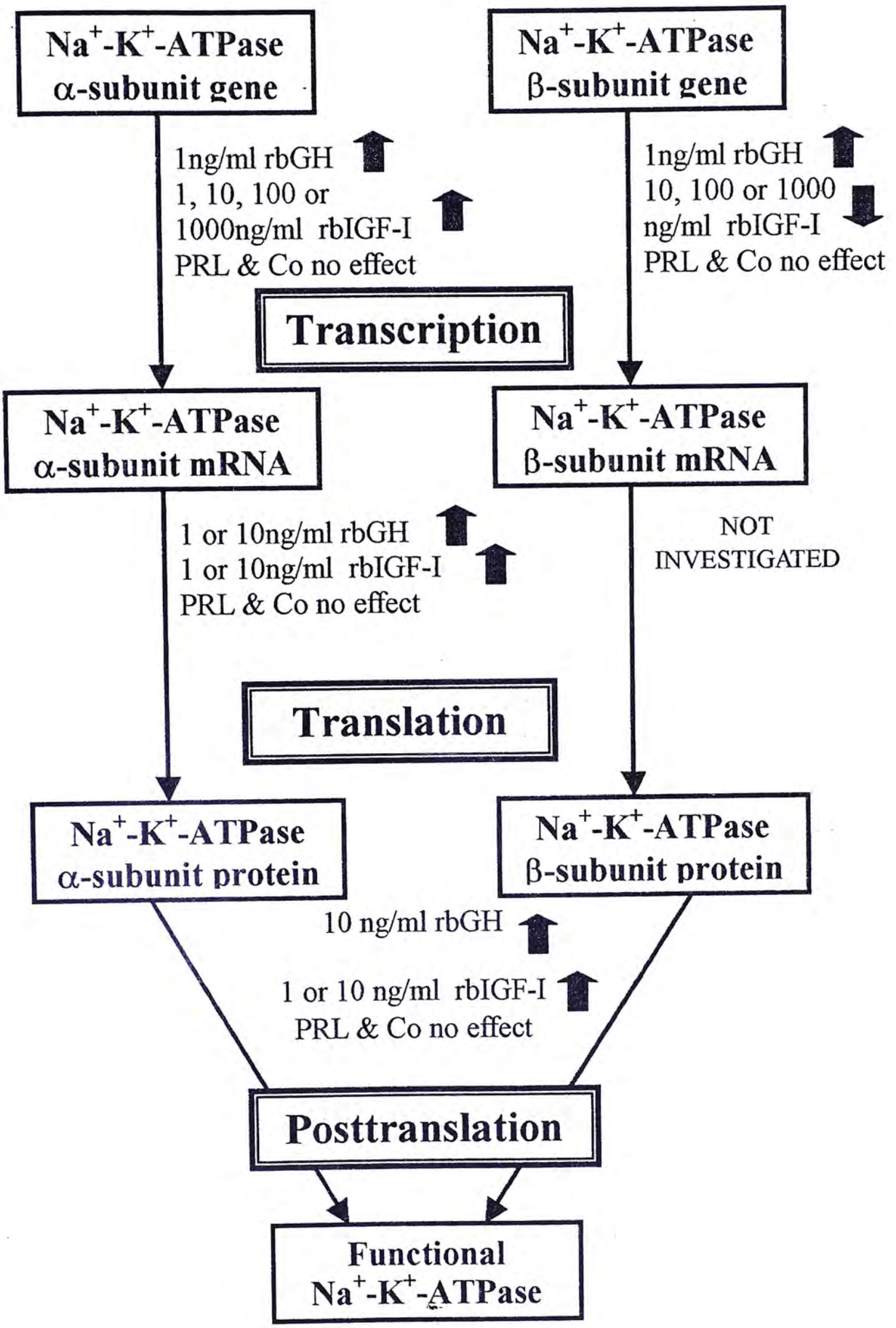


Figure 3.24. A schematic diagram summarizing the main findings of the effects of hormones on Na^+-K^+-ATPase expression.

3.6. Conclusion

As a conclusion, the present study demonstrates that rbGH and rbIGF-I elevated Na^+-K^+-ATPase mRNA subunit expression and enzyme activity at lower doses (1 or 10ng/ml of hormone) but showed a decrease in efficacy at higher doses (100, 1000ng/ml), a phenomenon that is in line with the parabolic dose-response of *Sparus sarba* to both rbGH and rbIGF-I *in vitro*. Gill filament of silver seabream is sensitive to both rbGH and rbIGF-I *in vitro* but not for oPRL and cortisol.

rbGH increased the transcription process of α- and β-subunits significantly at a concentration 1ng/ml, induced the α-subunit translational process in a wider range (1-100ng/ml) and increased posttranslational process at 10ng/ml. rbIGF-I increased the transcriptional process of α-subunit at all doses but decreased β-subunit transciption at high doses (10, 100 or 1000ng/ml) significantly. It induces the α-subunit translational process at 1 or 10ng/ml and increased posttranslational process at 10ng/ml. The threshold concentration of rbGH or rbIGF-I is different for the induction effect on α-mRNA, α-protein and enzyme activity. Both rbGH and rbIGF-I activated transcription, translation and/or posttranslation of *Sparus sarba* branchial Na^+-K^+-ATPase and β-subunit is a definite requirement for the functioning of Na^+-K^+-ATPase.

The interaction of various hormones on the transcription, translation and posttranslation of branchial Na^+-K^+-ATPase is summarized in Figure 3.24.

Chapter 4

In vivo effect of salinity on branchial Na^+-K^+-ATPase expression in marine teleost *Sparus sarba*

4.1. Abstract

Sparus sarba (silver seabream) is a member of the Sparidae that has been shown to tolerate a wide salinity range. The effect of salinity on branchial Na^+-K^+-ATPase expression in *Sparus sarba* was studied by investigating the changes in subunits mRNA, protein levels and enzyme activity after acclimating the fish to different salinity levels (6ppt, 12ppt, 33ppt and 50ppt) *in vivo*.

For mRNA subunit analysis, total RNA from branchial tissue was analyzed by using silver seabream Na^+-K^+-ATPase α- and β-subunit cDNA clones as radiolabelled probes. β-subunit mRNA abundance of the fish acclimated to 33ppt and 50ppt was significantly higher than that of groups in 6ppt or 12ppt whereas no significant changes in α-subunit when comparing the groups. Both 12ppt and 33ppt groups showed 2 fold higher in abundance of Na^+-K^+-ATPase α-subunit protein than that in 6ppt. 50ppt acclimation further increased the α-protein subunit level (4 fold higher than 6ppt group). Only the group acclimated to 50ppt had significant elevation in branchial Na^+-K^+-ATPase activity (2.7-4 fold) compared with others.

The data suggest that salinity level induced a trend of increase in *Sparus sarba* branchial Na^+-K^+-ATPase expression at the transciptional, translational and posttranslational levels.

4.2. Introduction

The body fluid of teleost fish, which has an osmolality at levels between 11 and 14ppt (350-440 mOsm kg^{-1}) (Bone *et al.*, 1995; Alderdice, 1988), is separated by only a thin respiratory epithelium from the environmental media, which have an osmolality range of a few to 1000 mOsm kg^{-1}. Thus, an ability to osmoregulate is essential for their survival in either freshwater (FW) or seawater (SW) environments. A variety of osmoregulatory mechanisms operate to facilitate adaptation and the ultimate achievement of homeostasis through biochemical, physiological (Prunet *et al.*, 1985; Usher *et al.*, 1988; Seidelin *et al.*, 2000) and morphological alterations in branchial epthelium (Perry, 1997; Fernandes *et al.*, 1998) following salinity changes.

Silver seabream *Sparus sarba* is a member of the Sparidae. Members of the Sparidae, commonly referred to as sea bream, are marine fish that can be found in tropical and subtropical estuaries, and have been shown to tolerate a wide salinity range (Woo and Wu, 1982; Woo and Kelly, 1995; Sheaves *et al.*, 1999).

Several studies have either selectively studied or combined studies on electrolyte dynamics, substrate mobilization, and hormonal response of fish following abrupt salinity change (Jacob & Taylor, 1983; Madsen *et al.*, 1994, 1996; Morgan *et al.*, 1997). However, few studies have shown the regulation of Na^+-K^+-ATPase subunit expression under different salinity exposure. The increase of gill Na^+-K^+-ATPase activity during parr–smolt transformation of salmonids is ascribed to as a preadaptive mechanism to cope with salt load when the smolts enter the sea (Zaugg and McLain, 1970; Saunders and Henderson, 1978; McCormick and Saunders, 1987;

Staurnes *et al.*, 1993). Na^+-K^+-ATPase plays an important role both in hypo- and hyper-regulation (Kamiya and Utida, 1969; Hart *et al.*, 1981; McCormick, 1995; Jensen *et al.*, 1998) and many studies revealed that its activity showed a direct relationship with environmental salinity (Payan *et al.*, 1984; McCormick, 1995; Morgan *et al.*, 1997).

Iso-osmotic (ISO) adaptation is believed to be beneficial to aquaculture as it has been reported to enhance growth by minimizing the metabolic cost of osmoregulation (Narnaware *et al.*, 2000) and reorganization of metabolism which would allow preferential utilization of carbohydrate and lipid in order to keep the protein for growth (Woo and Kelly, 1995). Other than oxygen consumption rate, Na^+-K^+-ATPase activity is an indicator of osmoregulatory energetics. Gill Na^+-K^+-ATPase activity was the lowest in ISO, higher in FW and highest in SW in fish such as coho salmon (Morgan and Iwama, 1998). The purpose of this part of study was to examine the effect on Na^+-K^+-ATPase expression in gill of euryhaline silver seabream *Sparus sarba* acclimated to various salinities. Fish were acclimated to a strongly hyper-osmotic condition (50 ppt), normal seawater (33 ppt), iso-osmotic condition (12 ppt) and hypo-osmotic condition (6 ppt) and the expression profile of branchial Na^+-K^+-ATPase assessed.

4.3. Materials and methods

4.3.1. Overall experimental design

In this experiment, fresh gill filaments were cut from silver seabream acclimated to various salinities (6, 12, 33 and 50 ppt). Half of the treated gill filament was used for Na^+-K^+-ATPase mRNA subunits analysis by dot blotting, another half was used for Na^+-K^+-ATPase α-protein subunit and Na^+-K^+-ATPase activity analysis.

4.3.2. Fish preparation

Silver sea bream (*Sparus sarba*), weighing between 90-110g, were obtained from local sea cages (33ppt) and transferred to and held in recirculating seawater (SW, 33ppt) systems in The Marine Science Laboratory, The Chinese University of Hong Kong. Fish were randomly divided into three groups (n=15) and removed from stock tanks one week before starting the experiment. They were acclimated to culture conditions in aerated 1000-l experimental tanks equipped with recirculating filtration systems for 3 weeks. Temperature was held between 18 and 22°C and the fish were exposed to a natural photoperiod (11h L :13h D) and the salinity of the water during this period was 33ppt. Fish were fed *ad libitum* once daily with diets formulated by Woo & Kelly (1995). *Sparus sarba* were acclimated to various salinities (6, 12, 33 and 50 ppt) respectively for one month (n=7 per group). The salinity of appropriate experimental tanks was reduced to 6ppt via addition of aged (aerated in 1000-l holding tanks for 2-3 days prior to addition) tap water that was fully aerated and temperature regulated. Seawater was allowed to evaporate to 50ppt and the

hypersaline seawater was used to hold fish at 50 ppt for 3 weeks before experimentation.

4.3.3. Tissue sampling

Fish were killed by spinal transection and the heads were prepared for sampling of gill tissue. Gill tissues intended for RNA analysis, protein analysis and enzyme activity measurements, were not perfused and were removed from the fish immediately after decapitation.

Each gill arch was cut into two pieces, one for RNA analysis and another for protein analysis and enzyme assay, and were immediately frozen in liquid nitrogen and stored in deep freezer at -70°C for subsequent analysis.

4.3.4. RNA extraction, dot blot analysis, protein extraction, quantification, Na^+-K^+-ATPase activity, protein gel electrophoresis and immunoblotting (Western blotting)

Procedures of RNA extraction, dot blot analysis, protein extraction, quantification, Na^+-K^+-ATPase activity, protein gel electrophoresis and immunoblotting (Western blotting) were exactly the same as those described in Materials and Methods in Chapter 3.

4.3.5. Statistical analysis

All data are expressed as mean values & S.E.M. The groups were subjected to a one-way anova analysis of variance to test for significance followed by a Student–Neuman–Keuls multiple comparison test (SigmaStat statistical software, Jandel Scientific) to delineate significance between groups.

4.4. Results

4.4.1 Dot blot analysis of Na^+-K^+-ATPase mRNA subunits

For mRNA subunit analysis, total RNA from branchial tissue was analyzed by using silver seabream Na^+-K^+-ATPase α- and β-subunit cDNA clones as radiolabelled probes. There was a trend showing a rise in α-subunit mRNA level as the acclimation salinity increased (Fig. 4.1). However, the apparent differences are not statistically significant. β-subunit mRNA abundance of the fish acclimated in 33ppt and 50ppt significantly higher than the groups in 6ppt or 12ppt (~1.5 fold) (Fig. 4.2).

4.4.2 Analysis of Na^+-K^+-ATPase protein α-subunit

For protein subunit analysis, only Na^+-K^+-ATPase α-protein subunit was investigated. Both 12ppt and 33ppt groups showed 2 fold higher in abundance of α-subunit protein than that in 6ppt. 50ppt acclimation further increase the α-protein subunit level (4 fold higher than 6ppt group) (Figs. 4.3 & 4.4).

4.4.3 Analysis of Na^+-K^+-ATPase activity

Sparus sarba acclimated to higher level of salinity generally showed higher functional Na^+-K^+-ATPase activity. However, only the group acclimated to 50ppt revealed significant difference when compared with other groups. The 50ppt group displayed a 2.7-4 fold elevation in branchial Na^+-K^+-ATPase activity over those of fish acclimated to 6-33ppt (Fig. 4.5).

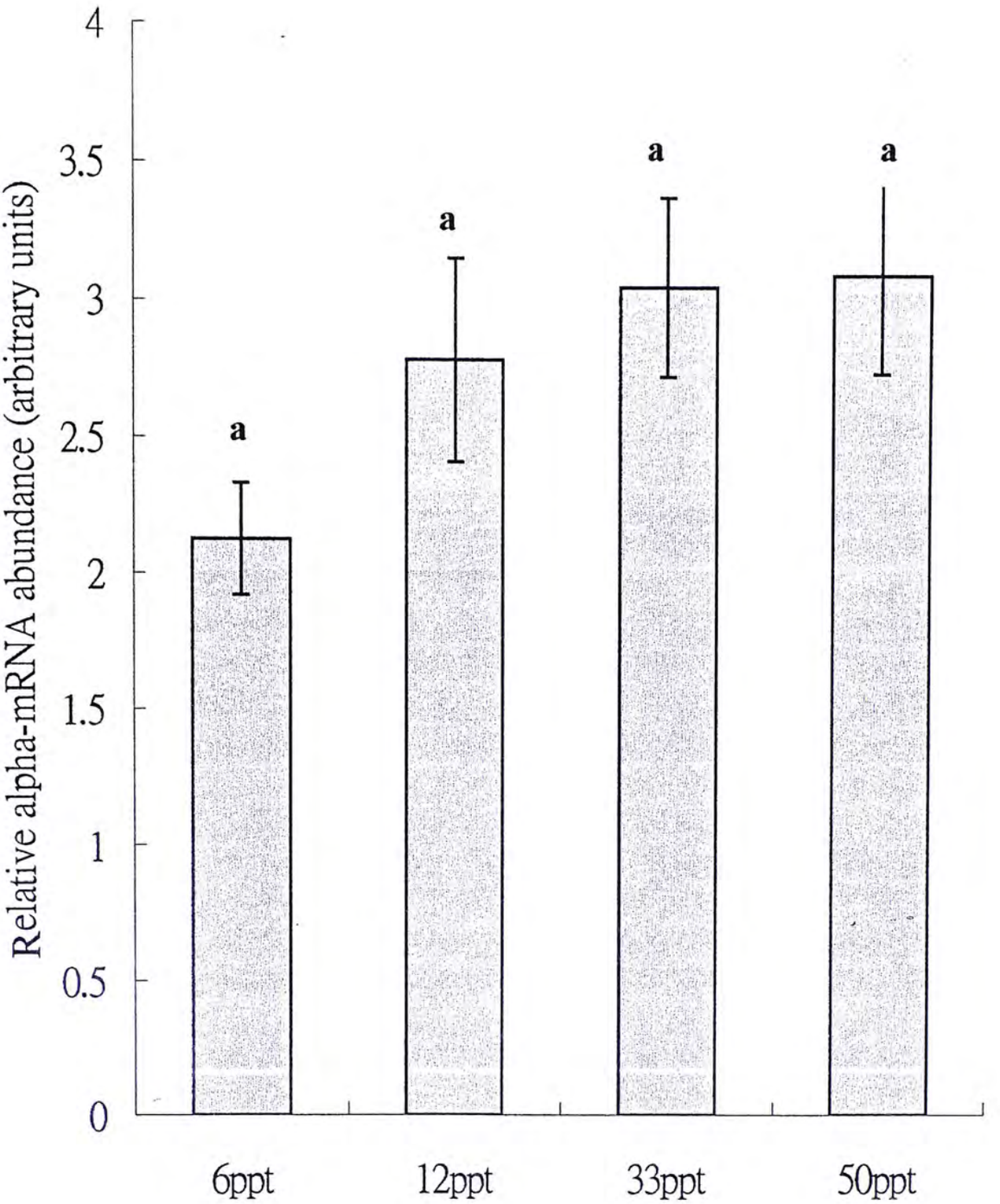


Figure 4.1. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-mRNA abundance after acclimated in 6, 12, 33, 50ppt. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-mRNA level (n=7-8).

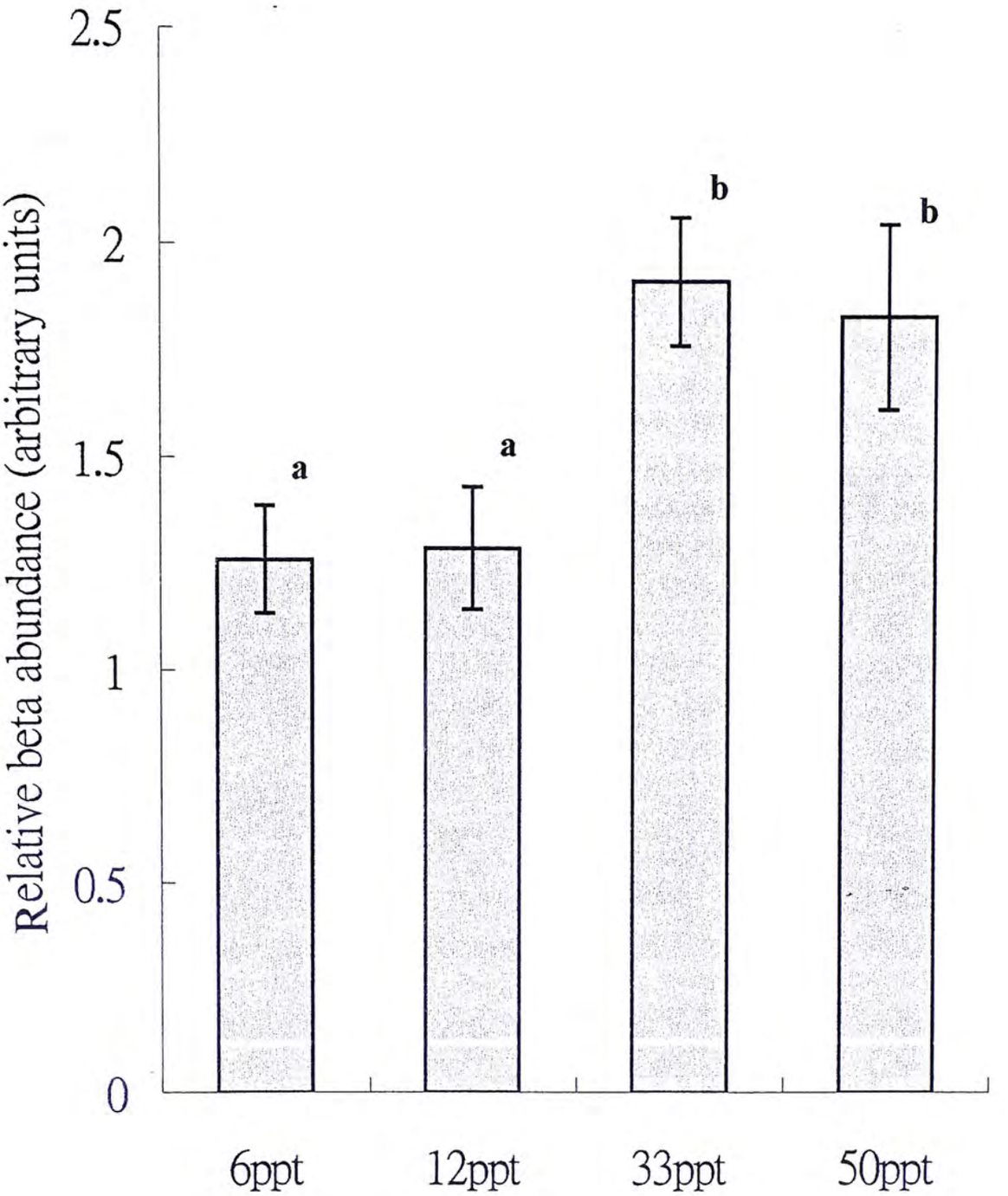


Figure 4.2. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase β-mRNA abundance after acclimated in 6, 12, 33, 50ppt. Values are means ± S.E.M. branchial Na^+-K^+-ATPase β-mRNA level (n=8). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

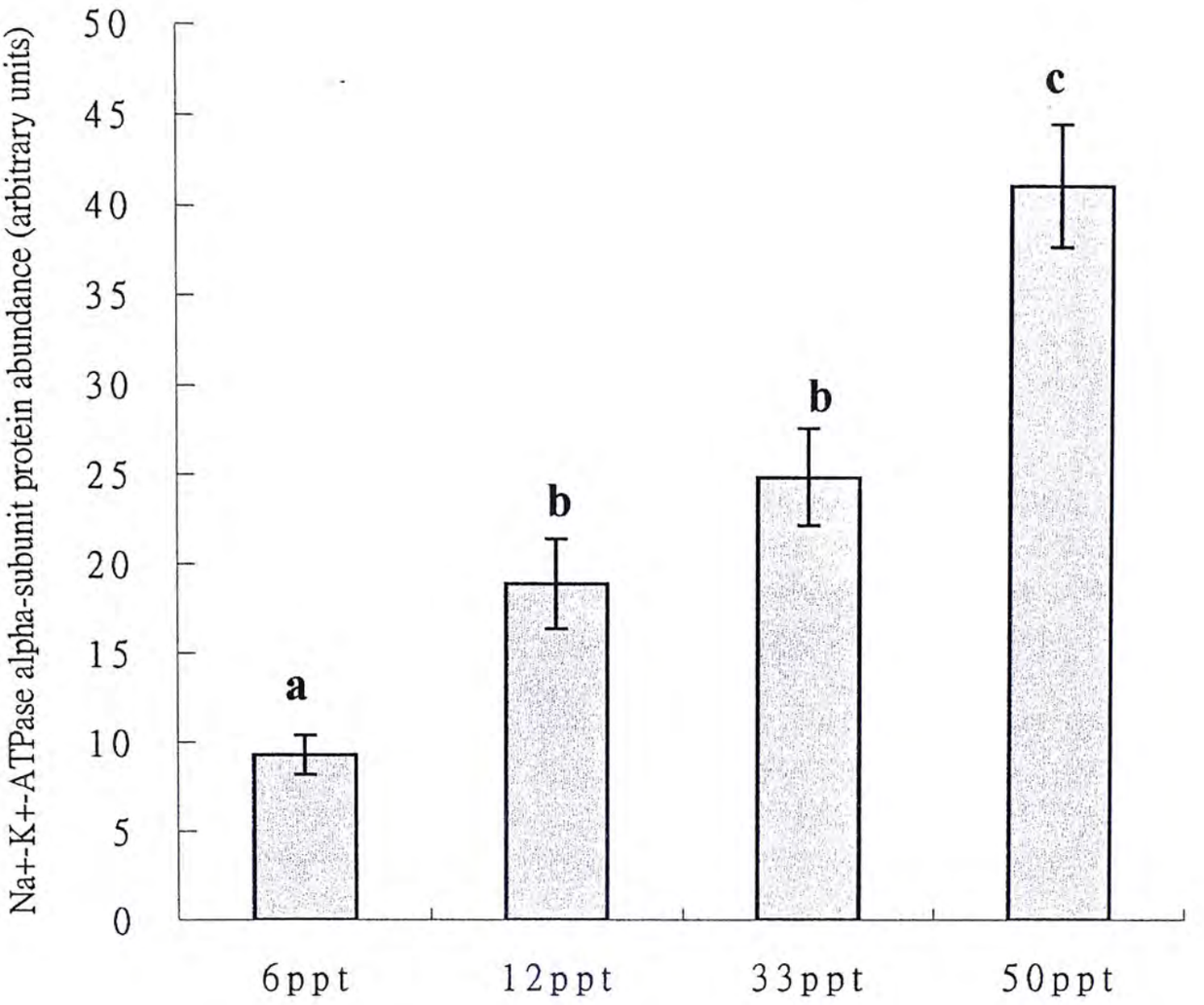


Figure 4.3. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase α-protein abundance after acclimated in 6, 12, 33, 50ppt. Values are means ± S.E.M. branchial Na^+-K^+-ATPase α-protein level compared to 6ppt (n=6). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

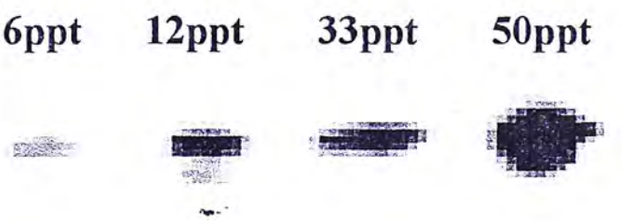


Figure 4.4. Representative of western blot of different salinity level acclimated *Sparus sarba* branchial tissues. The blot was probed with monoclonal α5 antibody (antimouse) specific to Na^+-K^+-ATPase α-protein subunit.

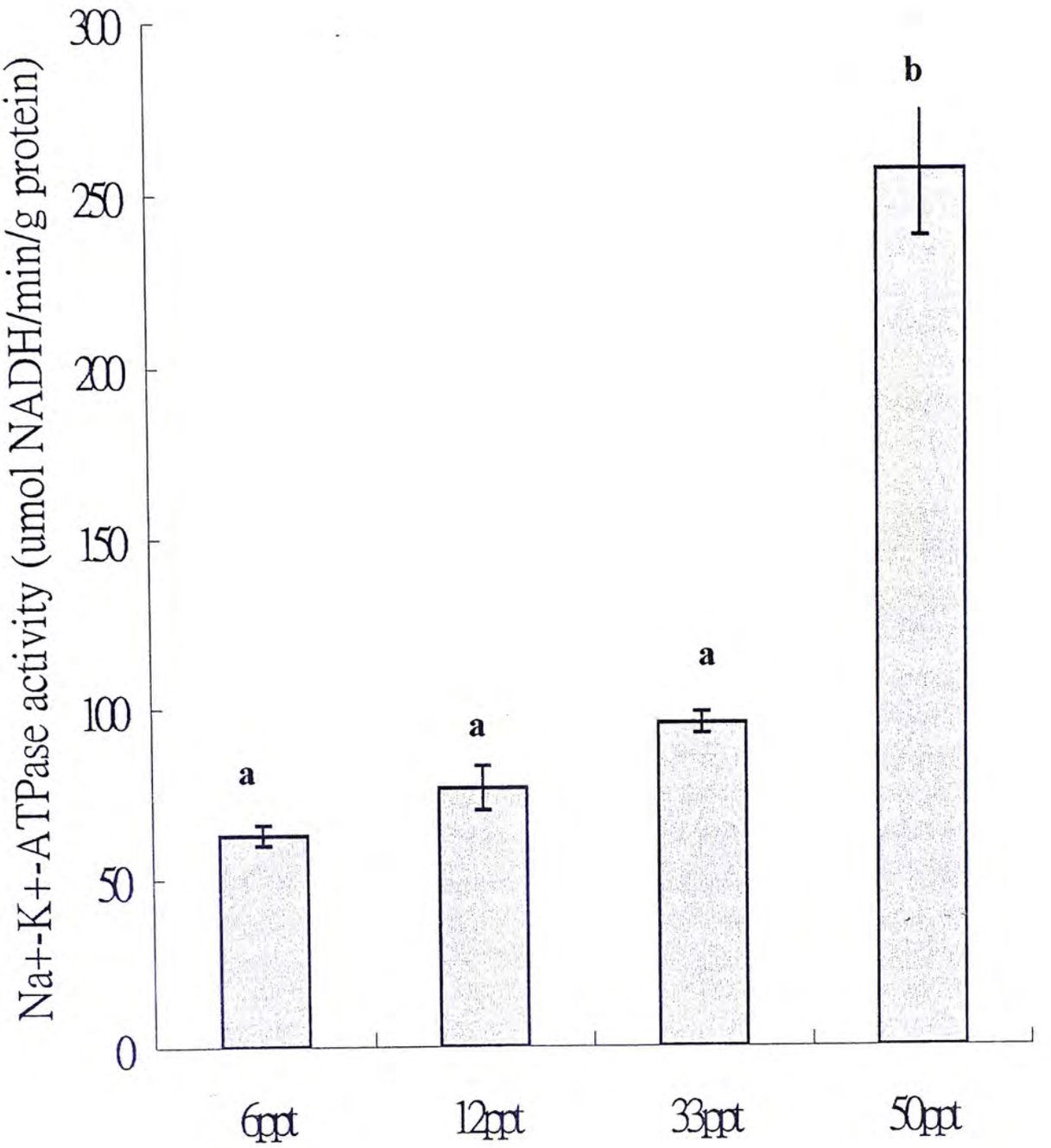


Figure 4.5. *Sparus sarba*: Relative branchial Na^+-K^+-ATPase activity after acclimated in 6, 12, 33, 50ppt. Values are means ± S.E.M. branchial Na^+-K^+-ATPase activity (n=6). Values with different alphabets are significantly different from each other ($P<0.05$). Student-Newman-Kuels Multiple Comparison.

4.5. Discussion

There was a trend showing a general elevation in α-subunit mRNA level as the acclimation salinity increased but the differences are not statistically significant in the current study. β-subunit mRNA abundance of the fish acclimated to 33ppt and 50ppt was significantly higher than those of the groups in 6ppt or 12ppt indicating that SW induced Na^+-K^+-ATPase β-subunit transcription. As the level of β-subunit mRNA in 33ppt was more or less the same as that of the group in 50ppt, it is possible that SW induction of Na^+-K^+-ATPase β-subunit transcription is dependent on the basal osmolality level but not on increase in external medium osmolality. The present study revealed that *Sparus sarba* branchial Na^+-K^+-ATPase β-subunit mRNA is more sensitive to SW than α-subunit mRNA. In other studies, the amount of Na^+-K^+-ATPase α-subunit mRNA in gill tissue increased with the level of environmental salinity in tilapia (Hwang, 1998; Feng *et al.*, 2002) and after abrupt transfer to 25ppt SW in brown trout (Seidelin *et al.*, 2000).

For protein subunit analysis, the amount of branchial Na^+-K^+-ATPase α-subunit protein increased with the level of environmental salinity. 12ppt and 33ppt groups showed 2 fold higher in abundance of α-subunit protein than that in 6ppt group. 50ppt acclimated group with ~4 fold increased in the α-protein subunit level when compared to 6ppt group. The data showed obviously higher Na^+-K^+-ATPase α-subunit protein expression in hyperosmotic than in hypoosmotic condition, suggesting that high external osmolality induces increased translation and/or stability of Na^+-K^+-ATPase α-protein subunit. The present results are similar to study tilapia when the

amount of Na^+-K^+-ATPase α-subunit in gills of SW-adapted fish was significantly higher than that in FW-adapted ones (Hwang, 1998).

In the present study, functional Na^+-K^+-ATPase activity increased as the level of salinity increased, although only the activity at 50ppt was significantly different (2.7-4 fold elevation) from those of other groups. Similar results in euryhaline teleosts have been demonstrated in several studies (*Oncorhynchus mykiss:* Madsen and Naamansen, 1989; *Gillichthys mirabilis:* Yoshikawa *et al.*, 1993). In *Sparus sarba,* branchial chloride cell numbers and exposure areas were elevated in both 50 and 6‰ environments, however, branchial Na^+-K^+-ATPase activity was generally higher in 50 and 33 ppt and exhibited a declining trend in 12 ppt and 6 ppt. 0ppt resulted in a marked elevation in branchial Na^+-K^+-ATPase activity (Kelly *et al.*, 1999b). Gradual transfer to seawater (using three steps of 9, 20, 28ppt) had a stimulatory effect on gill Na^+-K^+-ATPase activity (Johnston and Cheverie, 1985; Fuentes *et al.*, 1997; Seidelin *et al.*, 2000). Salinity dependency of gill Na^+-K^+-ATPase was also shown in the salmonid group where gill Na^+-K^+-ATPase activity and chloride cell density increase in proportion with increased external salinity (*Salmo salar*: McCormick *et al.*, 1989). The increase of gill Na^+-K^+-ATPase activity during parr–smolt transformation of salmonids has been ascribed to as a preadaptive mechanism to cope with salt load when the smolts enter the sea (Zaugg and McLain, 1970; Saunders and Henderson, 1978; McCormick and Saunders, 1987; Staurnes *et al.*, 1993). Tilapia *Oreochromis mossambicus* also displayed a marked reduction in branchial Na^+-K^+-ATPase activity when held in either FW or environments of 10‰ or 33‰ when compared with hypersaline environment (Jurss *et al.*, 1984, Kultz and Jurss, 1991). Compared with branchial Na^+-K^+-ATPase activity in saline-treated fish, those acclimated to 6ppt

exhibited lower Na^+-K^+-ATPase activity. This may be due to increase in size and density of chloride cells at higher salinities (Ayson *et al.*, 1994; McCormick, 1995).

The present study provides direct evidence that transcription, translation and posttranslation of branchial Na^+-K^+-ATPase in seabream are salinity dependent. Higher level of external osmolality induces transcription of β-subunit mRNA and translation of α-subunit protein and/or stability of the mRNA and protein subunit are part of the molecular mechanism responsible for increased Na^+-K^+-ATPase pump which corresponds to stimulation in its activity. Modulation of β-subunit expression is needed for the upregulation of functional Na^+-K^+-ATPase. Similarly, increased in α-subunit mRNA corresponding to the increase in Na^+-K^+-ATPase activity was observed in tilapia (Hwang, 1998).

The increase of gill Na^+-K^+-ATPase activity can be partially related to the proliferation of chloride cells. Seawater exposure also induces the differentiation of accessory cells as well as the opening of cellular tight junctions to make the branchial epithelium more permeable (Karnaky, 1986).

The declining trend in the Na^+-K^+-ATPase subunits expression and activity with lower salinity environment observed in the present study suggests that alterations in the form, morphology and function of chloride cells in the branchial epithelium may play an important role in the ability of *Sparus sarba* to acclimate to low salinity conditions (Kelly and Woo, 1999b).

Different strategies have been used to enhance seawater adaptation of fish including hormonal treatments (Madsen, 1990a, b; Sakamoto *et al.*, 1993; Mancera *et al.*, 1993a). Na^+-K^+-ATPase levels in branchial tissue fluctuate depending on the environment and are partially related to the hormonal factors. Adaptation to seawater in euryhaline teleosts is thought to involve the development of ion secretion pathways in the chloride cells of the gill epithelium under the influence of GH, IGF-I, PRL and cortisol (Bern and Madsen, 1992; Mancera and McCormick, 1998).

The rise in α-transcript and subsequently Na^+-K^+-ATPase activity may be brought about by higher plasma GH level after transfer to increased salinity when compared with hypo-osmotic and iso-osmotic conditions (Pisam *et al.*, 1988). Studies have shown that levels of plasma GH increased wheareas levels of plasma PRL decreased when various salmonid species were exposed to SW (Young *et al.*, 1988; Sakamoto *et al.*, 1990, 1991, 1993; Sakamoto and Hirano, 1991; Yada and Hirano, 1992). Levels of pituitary GH mRNA increased in concert with a PRL mRNA decline following transfer of catfish from FW to SW (Tang *et al.*, 2001). Levels of IGF-I mRNA in the liver and the gill increased during smoltification (SW exposure) in coho salmon (Sakamoto *et al.*, 1995).

Several studies demonstrated that GH elevated Na^+-K^+-ATPase α-mRNA and activity *in vivo* (Madsen *et al.*, 1995; Madsen, 1990b). Chapter 3 of the present study demonstrated that both GH and IGF-I elevated *Sparus sarba* branchial Na^+-K^+-ATPase expression *in vitro*. Consequently, Na^+-K^+-ATPase expression was elevated. Increased GH production following SW transfer is likely to stimulate other aspects of metabolism (e.g. increased rates of protein synthesis). Work with salmonids suggests

that the initial rise in plasma GH levels is followed by a concomitant increase in the metabolic clearance rate of GH (Sakamoto *et al.*, 1990, 1991). The chronic elevation of GH cell activity in SW tilapia may help to explain the higher growth rates (Teskeredzic *et al.*, 1989; Kuwaye *et al.*, 1993; Ron *et al.*, 1995) despite lower feeding activity (Usher *et al.*, 1991) as observed in SW tilapia compared to FW fish. The volumetric, densitometric, and ultrastructural evidences suggested an activation of synthesis and release of PRL in *Sparus aurata* adapted to hypoosmotic environments (Mancera *et al.*, 1993a). PRL significantly reduced the level of *Sparus sarba* branchial α-mRNA in both SW and hypo-osmotic conditions *in vivo* (Deane *et al.*, 1999). Putting all the available evidence together, lower Na^+-K^+-ATPase expression will result with hypo-osmotic condition, as has been demonstrated in the present study. Salinity induction of hormonal production is also one of the strategies in fish hypo- or hyper-osmoregulatory.

The responses of teleost fishes to different rearing salinities affect growth and survival (Johnson and Katavic, 1986; Lee and Meu, 1986; Alderdice, 1988; Bone *et al.*, 1995). The metabolic cost of salinity stress is likely to be high, as was recently demonstrated by Morgan *et al.* (1997). The energy most probably essential for increased substrate utilization is not only to be found in the gills but also in other osmoregulatory organs such as the kidney. The extra costs for ionic regulation may reduce the energy available for growth unless the fish can compensate by increasing its feeding rate (Wootton, 1995). Maximum growth at occurred at around 10-20ppt in sea bass *Dicentrarchus labrax* (Johnson and Katavic, 1986) or around 12ppt in sea bream *Sparus sarba* (Narnaware *et al.*, 2000). A salinity of around 12-15ppt (or isoosmotic condition) was reported as the minimum stress environment for fish

survival, and fish at this salinity generally exhibited highest growth rates and protein efficiency ratios (Woo and Kelly, 1995). The growth enhancement is accompanied by lower in oxygen consumption, ammonia excretion rates, liver lipid levels and hepatic glucose-6-phosphatase activity (Woo and Kelly, 1995).

4.6. Conclusion

In the present study, in *Sparus sarba*, different levels of salinity were showed that not only affected Na^+-K^+-ATPase at the level of the pumping mechanism (Na^+-K^+-ATPase functional activity) but also acted on the molecular subunit expression at the transcriptional and translational level. β-subunit, in addition to α-subunit is essential for the functional activity of Na^+-K^+-ATPase. It showed that external NaCl acted as a stimulus on Na^+-K^+-ATPase expression.

Chapter 5

General discussion and conclusion

In this thesis, Na^+-K^+-ATPase expression of *Sparus sarba* was investigated and branchial tissue was used as an *in vitro* model for study. Although numerous studies have focused on the effects of hormones on Na^+-K^+-ATPase (McCormick, 1995; Kelly *et al.*, 1999c), it is still unknown how hormones regulate the expression of Na^+-K^+-ATPase molecular subunits. The present study is the first study on the endocrine regulation of molecular subunits level of branchial Na^+-K^+-ATPase in *Sparus sarba in vitro*. In the first part of the study, branchial Na^+-K^+-ATPase expression was studied after gill tissue has been treated with different hormones *in vitro*. In the second part, branchial Na^+-K^+-ATPase expression was investigated after fish were subjected to various osmotic stress *in vivo*. The investigations have been mainly focused on three main levels of Na^+-K^+-ATPase expression, i.e. Na^+-K^+-ATPase mRNA subunits, protein subunit and functional enzyme activity.

Following a 2-hour *in vitro* treatment with hormones, a direct hormonal regulation on branchial Na^+-K^+-ATPase expression was evident. It is obvious that rbGH and rbIGF-I exerted a stimulatory effect on Na^+-K^+-ATPase expression irrespective of mRNA subunits, protein subunit or the enzyme activity when physiological relevant levels (1-10ng/ml) of hormone were added. Induction of mRNA, protein subunits and activity levels at various doses suggested that different response thresholds existed among these parameters. Similar pattern of rbGH and rbIGF-I effects may be due to the existence of a GH-IGF-I interaction. IGF-I showed a greater effect on Na^+-K^+-ATPase expression than GH. IGF-I mediates most of the

functions of GH (Madsen and Bern, 1993; Sakamoto and Hirano, 1993). In the present study, it still could not be determined whether GH acts directly on Na^+-K^+-ATPase expression or whether there is initiation of endogenous IGF-I production for mediating GH functions. This can be investigated by comparing the branchial IGF-I level with and without GH treatment. The branchial Na^+-K^+-ATPase activity increased in parallel with mRNA and protein subunits. We can conclude that GH and IGF-I exert their effect on *Sparus sarba* branchial Na^+-K^+-ATPase mainly by directly regulating the synthesis or stability of both α- and β- mRNA subunits and protein subunit. It is still not fully understood whether the elevation of Na^+-K^+-ATPase expression is due to upregulation of Na^+-K^+-ATPase subunit synthesis or increase in the stability of the subunits. One way of clarifying this is to block the already synthesized (pre-existing) Na^+-K^+-ATPase and then evaluating the amount of newly-formed Na^+-K^+-ATPase after hormone treatment. In addition, the data suggested that the β-subunit, although not a catalytic domain, is essential for the functioning of Na^+-K^+-ATPase. The activity of Na^+-K^+-ATPase mainly depends on the regulation of β-subunit.

In vitro exposure to oPRL or cortisol, was found to have no significant effect on the regulation of branchial Na^+-K^+-ATPase subunits and activity under the conditions of the present study. Prolactin halved the level of branchial Na^+-K^+-ATPase α-mRNA in *Sparus sarba in vivo* (Deane *et al.*, 1999). Cortisol increased Na^+-K^+-ATPase activity and α-mRNA subunit levels in brown trout (Seidelin *et al.*, 1999) and also caused an increase in α-mRNA subunit in *Sparus sarba in vivo* (Deane *et al.*, 1999). Different results obtained between *in vivo* and *in vitro* experiments

within the same species suggest that exogenous oPRL or cortisol treatment alone could not evoke the regulation or expression of Na^+-K^+-ATPase.

The data reported in this thesis suggested that regulation of branchial Na^+-K^+-ATPase expression is multihormonally regulated and that the control of Na^+-K^+-ATPase (the main ion transporter) by the GH/IGF-I axis is a common feature among marine teleosts such as *Sparus sarba* and this is in line with the study of Mancera and McCormick (1998) on other euryhaline teleosts.

Enhanced Na^+-K^+-ATPase expression would be required to supply the driving force for osmoregulatory work. The observation that elevation of Na^+-K^+-ATPase expression occurred as the salinity level increased confirmed the classical role of Na^+-K^+-ATPase as a hypersaline adapting factor, thus coping with hypersaline stress (50ppt). Augmentation of GH level during seawater acclimation has been reported in several studies (Sakamoto and Hirano, 1993; Shrimpton *et al.,* 1995; Uchida *et al.,* 1998). This, together with the upregulatory effect of GH on Na^+-K^+-ATPase expression may be the underlying basis for the enhanced expression of Na^+-K^+-ATPase associated with increases in salinity.

However, further work is necessary to determine the mechanism of actions of these hormones and their interaction. For example, the level of pituitary or circulating GH and IGF-I levels in the osmoregulatory organs during hypersaline adaptation can be examined to see whether the elevation of Na^+-K^+-ATPase expression is due to the concomitant stimulation of the GH/IGF-I axis. Such studies, when completed, will

form a coherent framework on the mechanisms of hormonal regulation of branchial Na^+-K^+-ATPase expression in euryhaline teleosts.

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